

***TRICHINELLA* SPECIES AND *TOXOPLASMA GONDII* IN WOLVERINE (*GULO GULO*) FROM NORTHWESTERN CANADA**

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By

Rajnish Sharma

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ABSTRACT

Trichinella spp. and *Toxoplasma gondii* are important foodborne parasites world-wide, and are especially important in wildlife harvested for food and fur in Canada. Due to their top position in the food web as well as their scavenging behavior, large carnivores such as wolverines may be more exposed to these parasites through foodborne routes. Limited information is available on these parasites in wildlife from northwestern Canada, and such information is critical to control and prevent infections in animals and people. To conduct surveillance studies, the first and foremost step is to select samples suitable for detection of the parasites. I first compared tongues and diaphragms of wolverine harvested for fur for the detection of *Trichinella* species (Chapter 2), and found tongue could act as a better sampling site; therefore, further epidemiological studies on *Trichinella* used tongue tissues (Chapter 3). Using a double separatory funnel digestion method, we found larvae of *Trichinella* in the tongue of 78% (95% CI = 73–82) of 338 wolverine from the Yukon Territory. The mean intensity of infection was 22.6 ± 39 (SD, range 0.1–295) larvae per gram of muscle tissue. Species or genotypes of *Trichinella* circulating in the wildlife of the Yukon are not known. Using multiplex PCR, *Trichinella* T6 was revealed as the predominant genotype (76%), followed by *T. nativa* (8%); mixed infections with *Trichinella* T6 and *T. nativa* (12%) were observed. While confirming multiplex genotyping by DNA sequencing, I unexpectedly discovered a previously undescribed species of *Trichinella* in a wolverine from the Canadian North that appeared identical to *T. nativa* in multiplex PCR. Therefore, I investigated the phylogeny, geography and host range of this undescribed species in Chapter 4. My findings showed that this unknown species of *Trichinella*, to be designated initially as the T13 genotype, is in fact ancient, and putative sister of the lineages including *T. patagoniensis* and the remaining species [(sister-clade (T1 + T7) and sister-clade (T2, T6, T3, T8, T5, T9))] of the encapsulated group. A deep divergence for T13 indicates it is the oldest endemic lineage in North America and comparative historical biogeographic studies are necessary to resolve a history of distribution and evolution at high latitudes. Based on my field survey, T13 has only been found in wolverines, and further studies are required to understand the limits of geographic distribution and host range in North America. Overall, wolverines have a high prevalence, intensity, and diversity of *Trichinella* spp. This work demonstrated that wolverine can host at least 5 species of *Trichinella* (*Trichinella* T6, *T. nativa*, the new species – T13, *T. pseudospiralis*, and *T. spiralis*).

To find out the suitable sampling sites for detecting *T. gondii* in wolverines, I used magnetic capture-qPCR to compare tissue prevalence and intensity of DNA of *T. gondii* in heart and brain

(known predilection sites in other species) (Chapter 5). Tissue prevalence (16 positives) and infection intensity (1221 TEG) was higher in heart compared to brain (10 positives, 347 TEG); thus, heart was selected for the next objective (Chapter 6). I also showed that heart fluid (HF) and filter paper eluates (FE) of chest fluid performed equally well in Enzyme Linked Immunosorbent Assay (ELISA) and Indirect Fluorescent Antibody test (IFAT), and HF performed better with modified agglutination test (MAT). IFAT and ELISA had higher sensitivity, specificity, and accuracy compared to MAT (Chapter 5). I used this information to fulfill the next knowledge gap; lack of information on the status of *T. gondii* in the wildlife of northwestern Canada. I used an ELISA to determine the prevalence of antibodies, and a sequence-specific magnetic capture DNA extraction and real-time PCR (MC-qPCR) to detect DNA of *T. gondii* in wolverines in the Yukon. Antibodies to *T. gondii* were detected in 27.5% (95% CI 23.0-32.5%) of 338 heart fluid samples. Eighty-one wolverines were positive on both ELISA and MC-qPCR. Strong correlation between the results of ELISA and MC-qPCR was observed. Overall, prevalence of *T. gondii* in wolverines in the Yukon was lower than that reported in this species elsewhere in northern Canada, which corresponds with lower seroprevalence in people in the western vs central and eastern North American Arctic.

Due to their scavenging habits, apex position in the food chain and wide home range, wolverine hosted high prevalence, high larval intensity, and multiple species of *Trichinella*, and also showed moderate prevalence and intensity of *T. gondii* infections. Therefore, wolverine should be considered as a sentinel species for surveys for *Trichinella* spp. and *T. gondii* across their distributional range, and as potential indicators of human risk of exposure through foodborne routes.

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DEDICATION

Dedicated to

My mother, Smt. Pushpa Sharma

Wife, Ritu and daughter, Tanu

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LIST OF ABBREVIATIONS

Body condition index - BCI

Centre for Foodborne and Animal Parasitology - CFAP

Enzyme linked immunosorbent assay -ELISA

Filter eluates - FE

Foodborne Disease Burden Epidemiology Reference Group - FERG

Heart fluid - HF

Indirect fluorescent antibody test - IFAT

International Commission on Trichinellosis - ICT

Larvae per gram - LPG

Latex agglutination test - LAT

Magnetic capture- qPCR - MC-PCR

Modified agglutination test - MAT

Optical density - O.D.

Restriction Fragment Length Polymorphism - RFLP

Single copy orthologs - SCO

Tachyzoite equivalents per gram - TEG

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

1.1 *Trichinella* and *Toxoplasma*: the parasites

Zoonoses are the “diseases and infections naturally transmitted between vertebrate animals and man”[1]. There are more than 200 zoonoses (caused by bacteria, viruses and other parasites) that can be transmitted through food and these foodborne parasitic zoonoses are an important public health concern globally. The Foodborne Disease Burden Epidemiology Reference Group (FERG), a group established by the World Health Organization, estimated that 48.4 million cases and 59,724 deaths occurred in a year due to parasitic diseases; 48% of cases were of foodborne origin [2]. Of 24 most significant global foodborne parasites listed by the World Health Organization/United Nations Food and Agriculture Organization, *Toxoplasma gondii* ranked 4th, *Trichinella spiralis* ranked 7th, and other *Trichinella* spp. ranked 16th on the basis of public health importance [3]. Interestingly, on the basis of global trade significance, *T. spiralis* ranked 1st and *Trichinella* spp. 7th. In a systematic review, 65818 human cases of trichinellosis and 42 deaths from 41 countries were reported worldwide from 1986-2009 [4]. *Toxoplasma gondii*, one of the most successful parasites, infects about one-third of the world’s human population [5, 6].

1.1.1 *Trichinella*: General description

1.1.1.1 Biology, life cycle, transmission and geographical distribution

The life cycle of *Trichinella* spp. nematodes is direct and unique in that a single host acts as both definitive and intermediate host and there is no environmental stage (**Figure 1.1**). The life cycle of *Trichinella spiralis* is domestic and involves primarily pigs, whereas the life cycles of other *Trichinella* spp. often involve wildlife hosts. After consuming meat containing first-stage larvae (L1) of *Trichinella* spp., larvae are released by the action of gastric juice in the stomach. Larvae are passed into the small intestine, and undergo four molts within the epithelial wall before becoming adult worms. Male and female worms copulate, and females release newborn larvae, which travel via blood to reach different muscle tissues. In muscle tissue, each larva forms a nurse cell around it [7, 8]. Larvae in the muscle cell can live for years; for example,

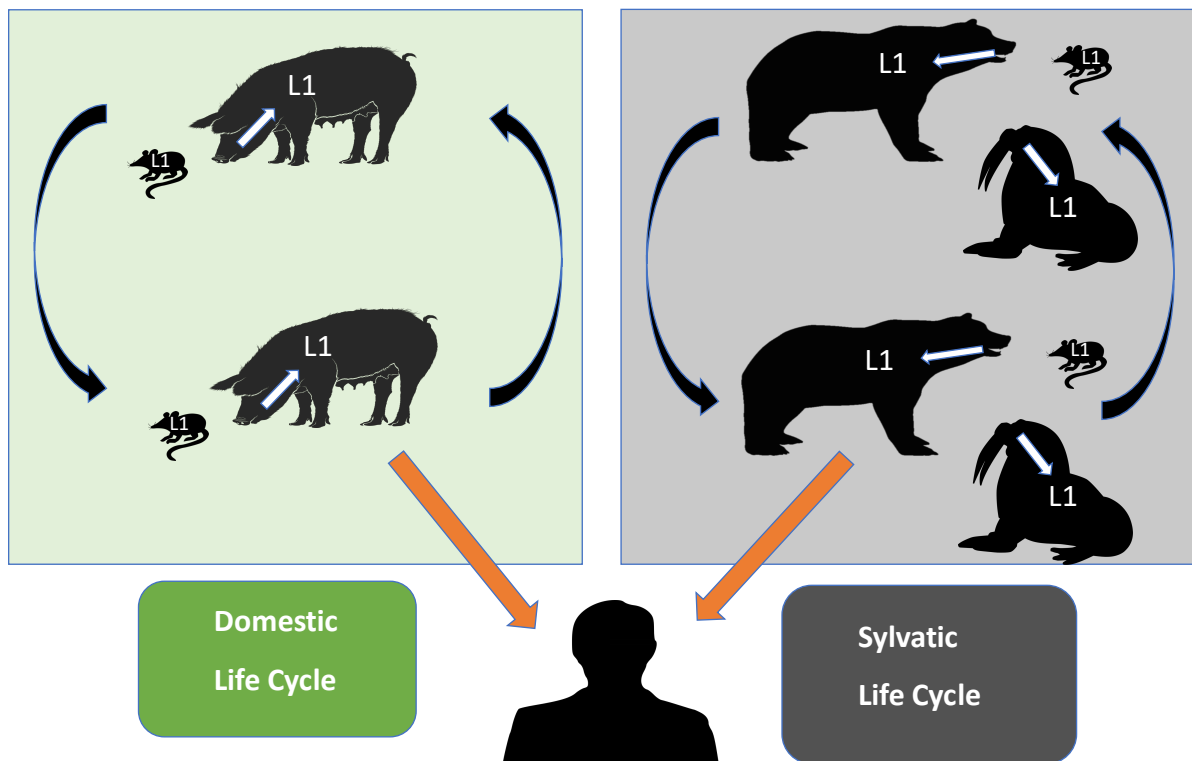


Figure 1-1 Life cycle and transmission of *Trichinella* spp.

(Pictures of pig, rat, walrus, bear and man were obtained from www.pixabay.com, and were free for use and no attribution was required).

documented reports showed larvae within muscles for up to 20 years in polar bears, and up to 40 years in humans [7, 9]. Infection of new hosts occurs after ingestion of meat containing L1. All *Trichinella* species and genotypes are zoonotic.

Based on the presence or absence of a thick collagen capsule around newborn larvae, species of the genus *Trichinella* are divided into two clades: encapsulated and non-encapsulated. The encapsulated clade consists of six species [*T. spiralis* (T1), *T. nativa* (T2), *T. britovi* (T3), *T. murrelli* (T5), *T. nelsoni* (T7) and *T. patagoniensis* (T12)] and three unnamed genotypes [*Trichinella* T6, T8 and T9], whereas the non-encapsulated group has three species [*T. pseudospiralis* (T4), *T. papuae* (T10), and *T. zimbabwensis*] [10, 11]. All *Trichinella* species and genotypes have been observed in mammals, while *T. pseudospiralis* is also reported in avian hosts, and *T. papuae* and *T. zimbabwensis* have also been documented in reptiles. *Trichinella spiralis* and *T. pseudospiralis* are cosmopolitan in their distribution due to the anthropogenic movements of pigs, and the role of migratory birds in dispersal, respectively [12, 13]. All other species are restricted to certain geographical areas. For example, *T. nativa* is commonly found in animals in the Holarctic region, and its southern boundary is between the isotherms -5 and -4°C in January [14]. Besides varied geographical distribution, species of *Trichinella* differ in adaptability to host species, and susceptibility to freezing.

1.1.1.2 Species of *Trichinella* in Canada

In Canada, five species of *Trichinella* have been reported in animals: *T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6 [15, 16]. In Canada, *T. spiralis* has been eradicated from commercially raised pigs, and is seldom reported in backyard pigs [16-20]. Wild animals in Canada are also considered free of *T. spiralis*, although infection has been observed in coyotes (*Canis latrans*) and red foxes (*Vulpus vulpus*) from Prince Edward Island, Canada [21]. A recent Canada-wide survey in multiple wild animal species did not find any cases of *T. spiralis*; however, four other species of *Trichinella* were reported, including *T. pseudospiralis* and *T. murrelli* in mountain lion (*Puma concolor*) from Vancouver Island, and northern-adapted species of *T. nativa* and *Trichinella* T6 in a wide range of wildlife hosts [16]. Both these species are considered important in terms of public health significance (due to their freeze resistant behaviour), and were reported as etiological agents in outbreaks of trichinellosis in people in Canada.

1.1.1.3 Wildlife hosts in the Canadian North

In the Canadian north, prevalence of *Trichinella* spp. infection varying from 1-77 % in several carnivore mammals has been reported [15, 16]. *Trichinella* spp. infections have been documented in Arctic fox (*Vulpus lagopus*), black bear (*Ursus americanus*), grizzly bear (*U. arctos*), lynx (*Lynx canadensis*), marten (*Martes americana*), polar bear (*U. maritimus*), red fox (*V. vulpus*), skunk (*Mephitis mephitis*), wolf (*Canis lupus*), wolverine (*Gulo gulo*), and walrus (*Odobenus rosmarus*) [16, 22-24]. Among these wildlife hosts, *T. nativa* and *Trichinella* T6 were most common, only *T. nativa* larvae were found in lynx, polar bear and walrus, and larvae in Arctic fox, red fox, skunk, and marten were not genotyped. In Nunavut and the Northwest Territories of northwestern Canada, circulating species or genotypes of *Trichinella* in wild animals are *T. nativa* and T6. To the best of my knowledge, three studies reported *Trichinella* spp. infection in wild animals from the Yukon [16, 23, 25]. Two of these studies, conducted more than 45 years ago, reported *Trichinella* in 71% (17/21) of grizzly bear of the Yukon [25] and 47% (60/127) of timber wolves of the Yukon and Northwest Territories [23]. Arctic fox, wolf and wolverines from the Yukon were also infected with *Trichinella* in a recent survey [16]. Another study conducted in polar bears from the Beaufort and Chukchi seas showed exposure to *Trichinella* using a serological test [26]. Previous research in this part of northwestern Canada did not involve geographically extensive sampling, determine risk factors associated with *Trichinella* spp. infection, or identify the species of *Trichinella* present. My thesis fills these knowledge gaps, and provides baseline data that would be helpful in future research.

1.1.2 *Toxoplasma gondii*: General description

1.1.2.1 Biology, life cycle, transmission and geographical distribution

Toxoplasma gondii infects almost all warm-blooded animals, including mammals and birds [27-29]. *T. gondii* has an indirect life cycle, and three infective stages: oocysts, tachyzoites and bradyzoites [28]. Oocysts are produced during the sexual reproduction of *T. gondii*, which occurs only in felid definitive hosts. Cats (domestic and wild) are the only known definitive host of *T. gondii*. Oocysts excreted in the feces of felids, if consumed by naive hosts, develop into tachyzoites (rapidly multiplying stage). Tachyzoites reach different tissues through the bloodstream, and develop into the slowly multiplying stage- bradyzoites. Tachyzoites and bradyzoites result from asexual reproduction that can occur in both definitive and intermediate hosts [5, 28]. A variety of domestic and wild animals can serve as intermediate hosts of *T. gondii*. The three main routes of

transmission are (*Figure 1.2*): (1) consumption of food or water contaminated with sporulated oocysts containing sporozoites, (2) consuming raw/undercooked meat of infected animals containing tissue cysts (bradyzoites), and (3) via transmission of tachyzoites vertically from mother to foetus. In addition, horizontal transmission of tachyzoites during blood transfusion and organ transplant can also occur in people [5, 27-31]. Based on genetic markers, and pathogenicity in mice, strains of *T. gondii* were initially classified into three clonal lineages: I, II and III [32]. Later, using multi-loci analysis of isolates of *T. gondii*, new genotypes have been added (atypical or non-archetypal isolates) [33]. Recent work suggests that the strains of *T. gondii* could be classified into six clades [34-36].

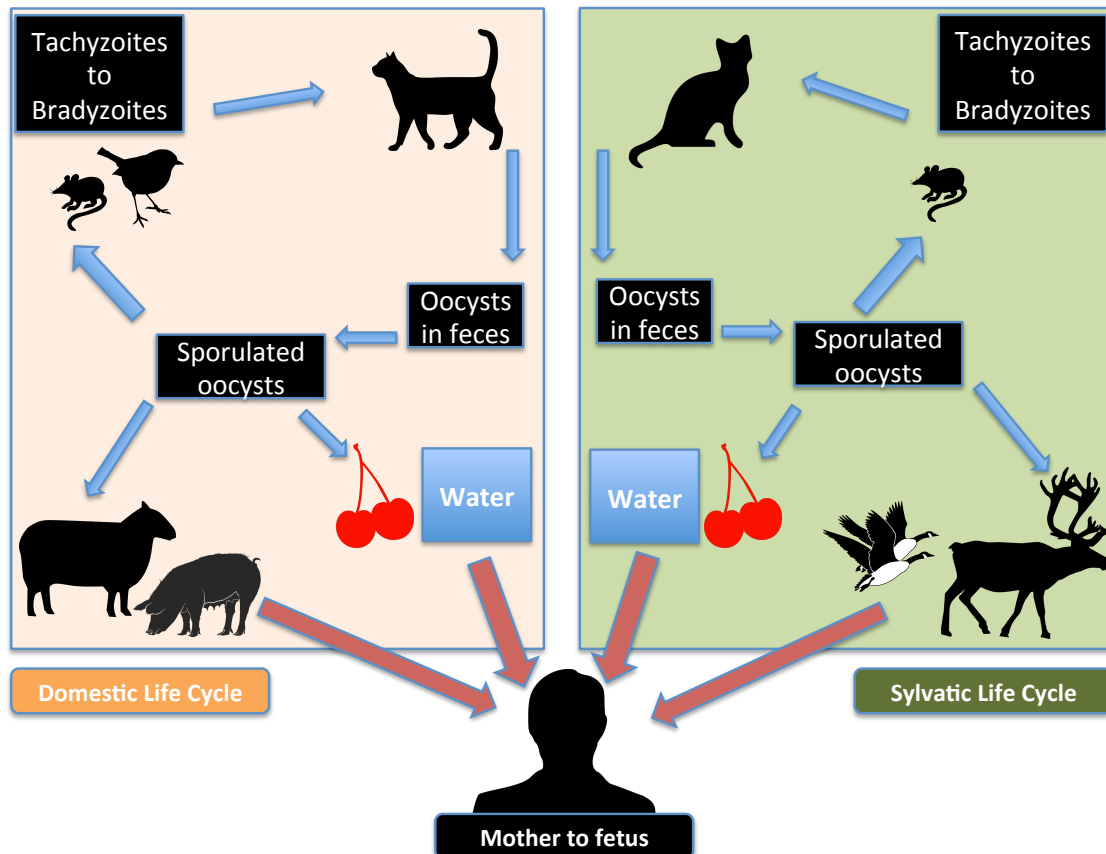


Figure 1-2 Life cycle and transmission of *Toxoplasma gondii*

(Pictures of cat, bird, pig, sheep, rat, cherries, caribou, goose and man were obtained from www.pixabay.com, and were free for use and no attribution was required).

1.1.2.2 *Toxoplasma gondii* in Canadian North

The life cycle of *T. gondii* is unknown in the Canadian North. Lynx are considered potential definitive hosts of *T. gondii* in northern Canada, but attempts to detect oocysts in their feces have not been successful. Absence of detection may reflect the short shedding period of oocysts in the cats [15]. Above treeline, lynx are rarely seen, and terrestrial migratory animals and/ migratory birds are assumed to play a role in transmission of *T. gondii* in regions above treeline [15, 37]. Exposure to *T. gondii* has been reported in wild animals including herbivores, carnivores and birds from the Canadian North. Almost all studies in wild animals from Canada detected antibodies to *T. gondii*, which indicate that animals have been exposed to *T. gondii* at one point in their lives. Prevalence of *T. gondii* antibodies varying from 2 to 42% were observed in mammals and migratory birds including muskox (*Ovibos moschatus*), caribou or reindeer (*Rangifer tarandus*), Arctic foxes (*V. lagopus*), lynx (*L. canadensis*), wolverine, harbour seal (*Phoca vitulina*), and ringed seal (*P. hispida*) [38-44]. Ross's Geese (*Chen rossii*) and Lesser Snow Geese (*C. caerulescens*) from Nunavut were also exposed to and infected with *T. gondii* [44, 45]. To the best of my knowledge, antibodies to *T. gondii* have been reported in the Porcupine caribou herd which migrate between the Yukon and Alaska, but there are no other reports of *T. gondii* in wildlife from the Yukon [40]. Detection of the *T. gondii* parasite or its DNA, as well as its genotypes, in the tissues of wild animals in Canada has rarely been attempted [46, 47].

1.2 Wolverines: the hosts

1.2.1 Wolverine (*Gulo gulo*): General Description

Wolverine (*Gulo gulo*) is a mesocarnivore, similar in appearance to a small bear; however, it is the largest terrestrial member of family Mustelidae in North America [48]. Wolverines are distributed across Eurasia and North America (mostly Alaska and northwestern Canada). The status of wolverine populations in Canada has been reviewed [49], and the number of wolverine in the Yukon is estimated to range between 3500–4000 [49]. Wolverine are sexually dimorphic, with males larger (on average 10% longer) and heavier (30%) than females. Females and males mature, on average, at the age of 15 months, and 15-27 months, respectively [50]. Their average life expectancy is 4-6 years [51]; animals less than 1 year, equal to one year, and equal to or more than 2 years are considered as juvenile, yearling and adult, respectively. Male wolverines have larger home range sizes than females [52]. The wolverine is omnivorous and its diet consists of carrion, small mammals, birds, and vegetation [52]; diet may vary with geographical locations and season. The winter diet of wolverines usually includes carrion; however, wolverines eat fresh prey

during summer [53]. Common sources of carrion are ungulates, especially muskox, moose, and caribou. Prey species include rodents, snowshoe hare, marten, birds and their eggs, and young ungulates (caribou) [53-60]. Wolves are the most common natural predator of wolverine [61]. Wolverines are fur-bearing mammals, and their valuable fur is harvested by communities and much-prized for trimming parkas.

1.2.2 Parasites of wolverines

Wolverines can be infected with a variety of parasites (**Figure 1.3**). Helminths that have been recovered in the intestines of wolverines from Alaska and the Northwest Territories included *Alaria* spp., *Taenia martis*, *T. twitchelli*, *Diphyllbothrium* spp., *Physaloptera* spp., *Baylisascaris devosi*, and *Molineus patens* [62, 63]. *Trichinella* spp. infection has been reported in wolverines across the world, with varied prevalence among countries (0% in Sweden, 30% in Russia, 50% in the USA) [64-66]. Canada wide, prevalence varied from 0 to 88% [16, 62, 67]; overall prevalence was 77% in wolverines (n=111) from the Yukon, British Columbia and Northwest Territories [16]. The highest prevalence of *Trichinella* spp. (88%) was reported in 41 wolverines from Nunavut [67]. Only 2 studies were conducted on *T. gondii* exposure in wolverines; sero-prevalence of 42% in 41 wolverines was documented in Nunavut [42], and none of 20 wolverines were positive for anti-*T. gondii* antibodies in BC [68]. Two species of *Sarcocystis* viz., *Sarcocystis kitikmeotensis*, and *S. kalvikus* have been described and named in wolverines from Nunavut, and overall prevalence of *Sarcocystis* spp. infection was 80% [69].

1.2.3 Significance of wolverines as sentinel species of *Trichinella* spp. and *Toxoplasma gondii* infections

Wild carnivores are considered as reservoir hosts of *Trichinella* and *T. gondii* infection, and can act as sentinel host species for detection of *Trichinella* spp. and *T. gondii* [70-74]. During a surveillance program, it is both time- and cost-effective to first study sentinel animal hosts rather than targeting multiple animal host species. Wolverine (*Gulo gulo*) exhibit characteristics of an ideal sentinel host species including the following (**Figure 1.4**): (1) measurable response (e.g. antibodies in blood; parasites in tissues): anti-*T. gondii* antibodies and *Trichinella* spp. larvae have been detected in wolverines [42, 67]; (2) Exposure by routes of transmission similar to those in the target hosts (people or game animals): wolverines are omnivores and can therefore contract *T. gondii* by consumption of oocysts in contaminated water or fruits/vegetation, and/or tissue cysts in infected carrion or prey. Wolverines, like other animals and people, can only become infected with *Trichinella* spp. by eating larvae in infected meat; (3) high levels of exposure: being in an

apex position in the food chain, wolverines bio-accumulate foodborne parasites such as *Trichinella* spp. and *T. gondii*; (4) earlier response than people/co-habiting animals (due to short life span): Wolverines have relatively short life spans (on average 5-6 years); and (5) adequate availability (population stability) for reliable survey, as wolverine carcasses are available from ongoing, legal harvesting of wolverines in some territories of Canada [51, 75, 76].

1.3 Impact of *Trichinella* spp. and *T. gondii* on wildlife and people

1.3.1 Impact of *Trichinella* spp. and *T. gondii* on wildlife

Symptoms due to *T. nativa* and *Trichinella* T6 in naturally infected wild animals are unknown. In experimentally infected grey seals (*Halichoerus grypus*), the major symptom noticed was anorexia until 17 days post infection, and then seals resumed eating [77]. Anorexia, diarrhoea, weight loss, and mild anemia were the clinical signs reported in experimentally infected raccoon dogs (*Nyctereutes procyonoides*) [78].

Clinical toxoplasmosis has been reported in wild animals including terrestrial carnivores e.g foxes [79, 80], wild boars [81], raccoons, *Procyon lotor* [82], and southern sea otters (*Enhydra lutris nereis*) [83]. Bilateral blindness and ataxia due to *T. gondii* infection were reported in free ranging mink [84].

1.3.2 Impact of *Trichinella* spp. and *Toxoplasma gondii* on people

In Arctic and subarctic regions of Canada, wildlife hosts play a major role in the transmission of *Trichinella* spp. and *T. gondii* infection to people and other animals. Due to traditional, cultural and subsistence values, people in northern Canada have frequent contact with wildlife, which could increase the risk of *Trichinella* spp. and *T. gondii* exposure of people.

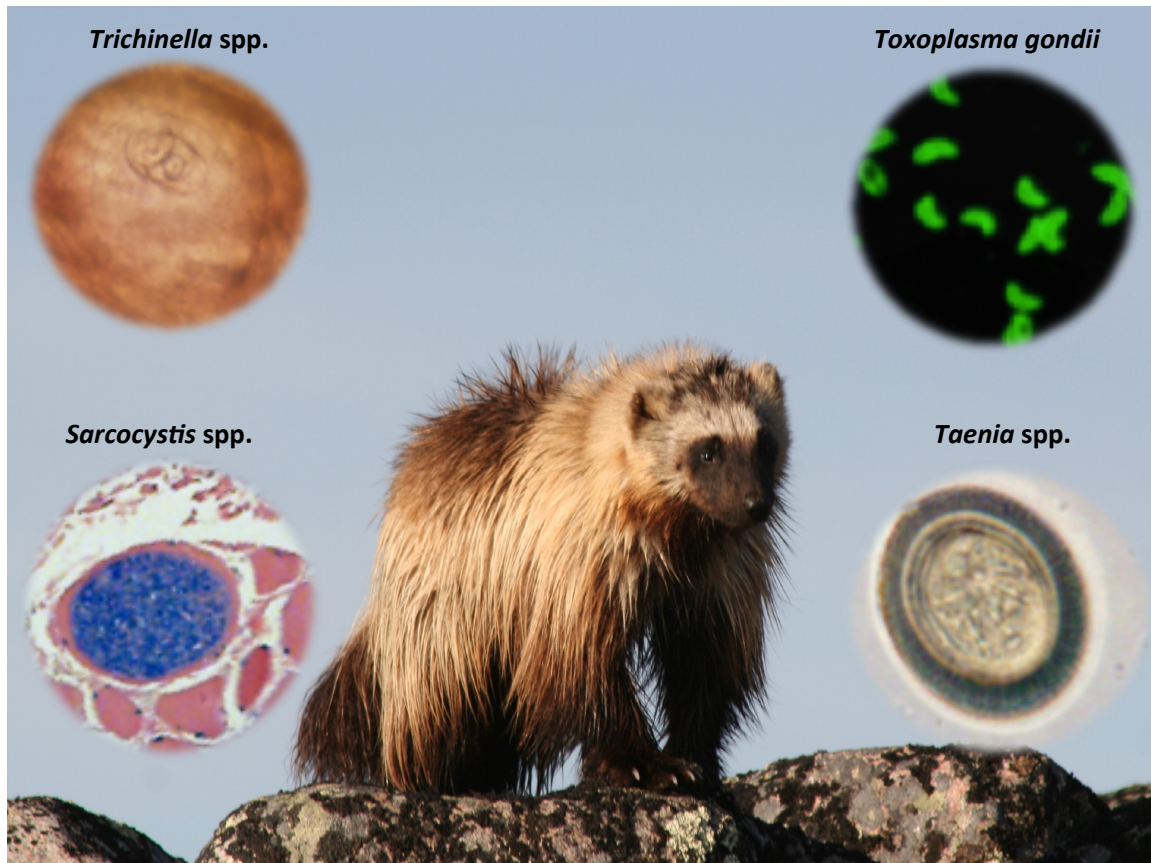


Figure 1-3 Parasites of wolverines

(Wolverine picture: credit to Gustaf Samelius, Snow Leopard Trust, Seattle, Washington 98103, USA).

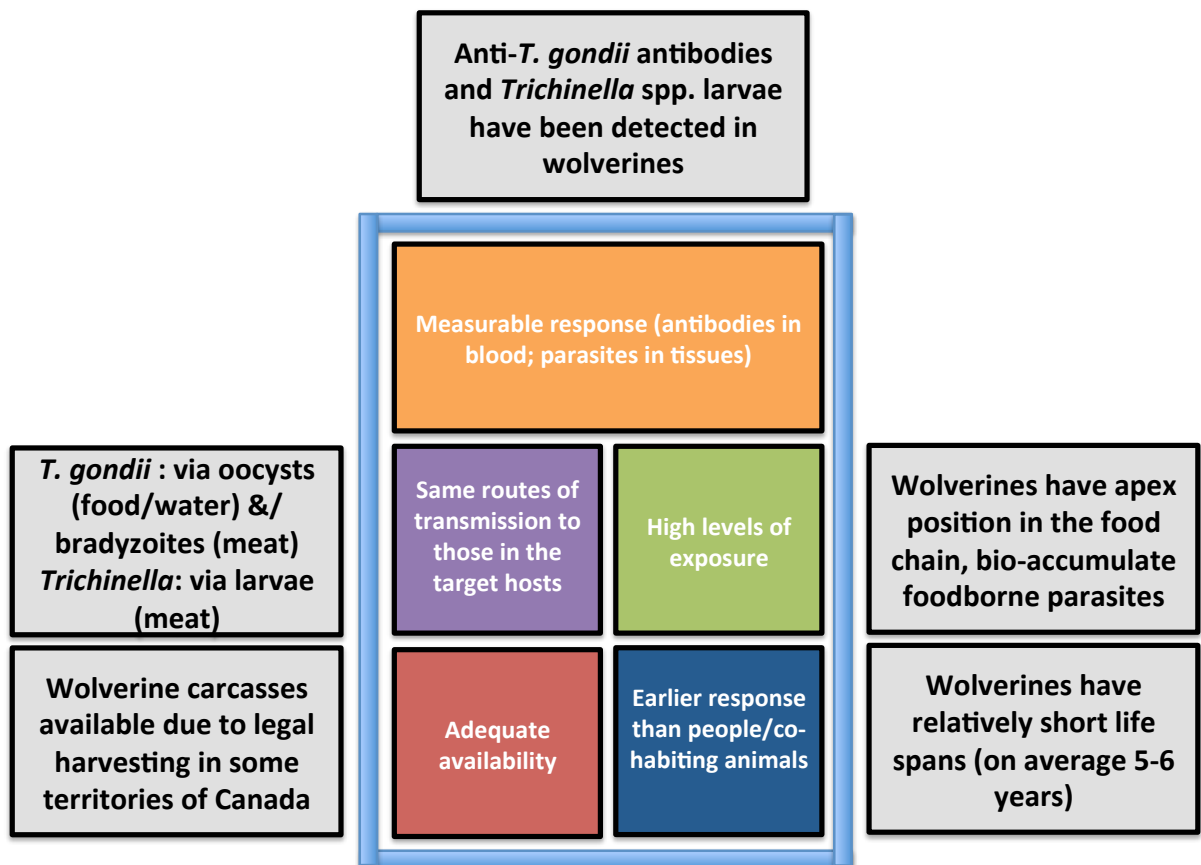


Figure 1-4 Wolverines as sentinels of *Trichinella* spp. and *Toxoplasma gondii*

Almost all recent human outbreaks of trichinellosis in Canada were associated with consumption of raw/improperly cooked game meat. Meat of bear and walrus were the most commonly reported animal sources in these outbreaks [85-88]. One of the largest outbreaks occurred in Saskatchewan, Canada, and 78 persons became infected after consuming meat of black bear infected with *T. nativa* [86]. Another recent outbreak that occurred in Ontario was linked with consumption of dried meat of black bear (*Ursus americanus*), and *T. nativa* was reported as the causative agent [87]. *Trichinella* T6 has also been identified as a cause of an outbreak in Ohio, United States, linked to consumption of bear meat in Canada [85]. Clinical manifestations due to trichinellosis in humans include abdominal pain, diarrhea, nausea, vomiting, fever, facial or periorbital edema, myalgia and skin rashes [11, 89]. However, rarely, cardiac, pulmonary, and nervous system complications may occur [90].

Acute toxoplasmosis (including retinal lesions) after consumption of raw venison was reported in three US hunters [91]. An outbreak of congenital toxoplasmosis occurred in northern Quebec, Canada, and was statistically linked to skinning of animals and consumption of raw dried caribou meat [92]; this region also has the highest seroprevalence of antibodies to *T. gondii* (60%) in any region of North America [93]. In addition to foodborne outbreaks, an outbreak of toxoplasmosis in British Columbia, Canada was linked to contamination of municipal drinking water with feces of cougars (*Felis concolor vancouverensis*) as well as domestic cats [94]. *T. gondii* usually does not cause any clinical disease in immunocompetent people, but can lead to pregnancy loss in women exposed acutely during pregnancy, and serious clinical manifestations (ocular and neurological) in fetuses of pregnant women exposed acutely during pregnancy, and in immunosuppressed people e.g., AIDS [28].

1.4 Methods for detecting *Trichinella* and *Toxoplasma gondii* in wildlife

1.4.1 Methods of detection of *Trichinella* spp.

1.4.1.1 Indirect methods for detecting *Trichinella* spp.

Serological methods are indirect measures of infection status, and detect anti-*Trichinella* antibodies in the serum or meat juice of animal tissues. Serological examination can be performed either ante- or post-mortem [95]. Enzyme linked immunosorbent assay (ELISA) is the most commonly used serological method (using E/S antigen or TSL-1 antigen) for detection of anti-*Trichinella* antibodies [96, 97]. Serum is considered the best matrix for serological diagnosis, but meat juice from slaughtered animals can also be used [96, 98, 99]. Serological methods can only

suggest exposure to *Trichinella*, and false negatives can result if animals are tested in early stages of infection [100]. For food safety purposes, serological methods cannot substitute direct detection methods during meat inspection of carcass as per the International Commission on Trichinellosis (ICT) [101].

1.4.1.2 Direct methods for detecting *Trichinella* spp.

Trichinoscopy, histology, and artificial digestion are methods of direct detection of active infection with *Trichinella*. Trichinoscopy is largely considered an obsolete technique due to low sensitivity, and artificial digestion is the recommended method for detection and recovery of *Trichinella* larvae in muscles of infected animals [11]. Despite higher sensitivity of the artificial digestion method, for surveys where mean larval intensity (measured as larvae per gram, LPG) is expected to be low, large sample sizes are often needed [101]. Unlike many helminths, adult nematodes are only transiently present in the intestine of the host, and there are no shed stages; therefore, direct detection is limited to larvae in muscles. As well as sample size, selection of sampling sites in the host is also a key factor, as different muscles are predilection sites in different hosts [11]. Predilection sites for *Trichinella* larvae are those muscles that consistently contain the most larvae in infected animals [16]. These sites are well defined in some domestic animals, such as horses (*Equus caballus*) and pigs (*Sus scrofa*), and usually include metabolically active tongue, diaphragm and masseter muscles [134]. Information on muscle predilection sites of *Trichinella* spp. is available for large carnivores (e.g. foxes [*Vulpes* spp.], raccoon dogs [*Nyctereutes procyonides*]) but not for wolverines. Such knowledge would be helpful for determining the preferred sampling site for epidemiological surveillance of *Trichinella* infection in wolverines. My thesis will expand knowledge about the predilection site of *Trichinella* spp. in the wolverines.

1.4.1.3 Identification of species or genotypes of *Trichinella*

Larvae of species of *Trichinella* cannot be differentiated based on their morphology, thus identification of species or genotype of *Trichinella* larvae can only be done by molecular methods e.g., conventional PCR, multiplex PCR, and real time PCR [102-105]. Multiplex PCR is the most commonly used molecular method; it is inexpensive and rapid, and targets internal transcribed spacers ITS1 and ITS2 and the expansion segment V region of the rRNA [102].

1.4.2 Methods of detection of *T. gondii*

1.4.2.1 Indirect methods for detecting *T. gondii*

Serological methods are indirect methods that indicate only exposure to, rather than active infection with, *T. gondii* [106]. For serological tests, serum is the specimen of choice, but it is not

always feasible to obtain sera from wild animals [107-109]. Several substitutes for sera in wild animal surveys primarily include blood, blood or body cavity fluid on filter paper, and meat juice [45, 74, 107, 108]; however, each specimen has its own merits and demerits. For example, collecting blood or cavity fluid on filter paper that can be air dried is preferred in remote areas where centrifugation and freezing are not available [108, 109]. Meat juice and filter eluate have been used in several wildlife studies for detection of anti-*T. gondii* antibodies [45, 74, 107, 108]. My thesis compares performance of meat juice vs. filter eluate in wolverines.

Previous serological studies have used the modified agglutination test (MAT), enzyme-linked immune sorbent assay (ELISA), indirect fluorescent antibody test (IFAT), latex agglutination test (LAT) and indirect hemagglutination test (IHA). Each test has its own merits and demerits; for example, MAT does not require species-specific conjugate and specialized equipment; however, MAT can be laborious, time consuming and subjective. ELISA and IFAT are rapid and less laborious, but these tests need special equipment and species-specific conjugate [110]. For the detection of anti-*T. gondii* antibodies, commercially kits are generally available, but not validated for use in wildlife. My thesis compares performance of ELISA, MAT, and IFAT kits with each other, and to detection of DNA in tissues. These results will be helpful for researchers to select tests for surveys on *T. gondii* in wildlife.

1.4.2.2 Direct methods for detecting *T. gondii*

Direct methods detect parasites or DNA in tissue, reveal if the animal is actually infected, and include bioassays, tachyzoite culture, immuno-histochemistry and molecular techniques like PCR [110]. Cat and/or mouse bioassay is the gold standard test. Cat bioassay has the highest sensitivity; however, it is expensive, time consuming, and has ethical issues [29, 110, 111]. Other direct detection methods include conventional and real time PCR assays. Conventional PCR methods have been developed for targeting multi-copy (B1, 529 bp repeat element) and single copy (SAG1, SAG2, GRA1) genes of *T. gondii* [110, 112-114]. Real time PCR methods are rapid, easy, and quantitative. Recently, magnetic capture- qPCR (MC-PCR) [115] has been successfully used to detect *T. gondii* DNA in wild animals [74], and showed higher sensitivity than conventional PCR methods [116]. Predilection site for *T. gondii* is also important for the sensitivity of a test; for example, the heart and brain are the most common predilection sites for this parasite in many species [106, 111, 117, 118]. Such information is lacking for wolverines, and my thesis would fill this knowledge gap.

1.4.2.3 Identification of genotypes of *T. gondii*

For identifying the genotypes of *T. gondii*, several methods have been developed viz PCR-RFLP (unilocus, multilocus, multiplex multilocus), RAPD-PCR, microsatellite analysis, multilocus sequence typing and high resolution melting (HRM) analysis [110, 114, 119-123]. PCR-RFLP targeting single locus is simple and inexpensive, but may lead to misidentification due to inability to capture complex allelic combinations [124]. Another disadvantage is requirement of large amount of DNA. Multiplex multi-locus nested PCR RFLP does require limited amount of DNA [34]. RAPD-PCR is a quick, simple, efficient, and requires highly pure DNA [34, 110, 125].

1.5 Objectives and hypotheses

My thesis focuses on two important food-borne parasites, *Trichinella* spp. and *Toxoplasma gondii*, in wolverines from northwestern Canada (**Figure 1.5**): The objectives of the thesis are:

1. To determine the predilection sites of *Trichinella* species in the wolverines.
 - a. I hypothesize that mean larvae per gram will vary between tongue and diaphragm
2. To measure the prevalence, intensity and genotypes of *Trichinella* spp. in the wolverines
 - a. I hypothesize that prevalence and intensity will be high in wolverine from northwestern Canada, based on their apex position in the food web and scavenging lifestyle
 - b. I hypothesize that there will be a diversity of *Trichinella* spp./genotypes in wolverine, and that freeze-adapted northern strains will dominate.
 - c. I hypothesize that age (adult than juvenile wolverines), sex (male than females), and body condition index (higher than lower BCI), will be the risk factors for the *Trichinella* spp infection prevalence and intensity in wolverines
3. To determine the phylogeny, geography and host range of a previously unrecognized species of *Trichinella*

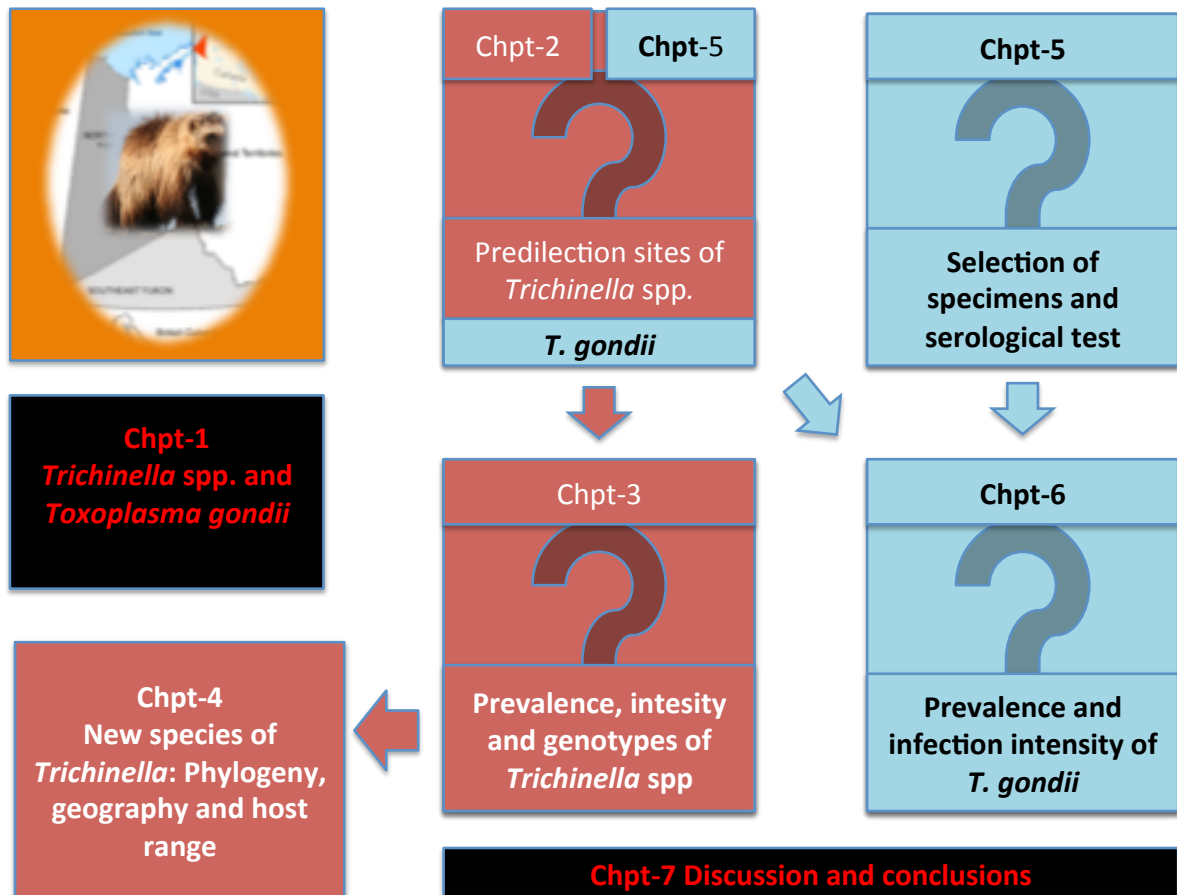


Figure 1-5 Framework to determine the status of *Trichinella* and *Toxoplasma gondii* from northwestern Canada.

(Wolverine picture: credit to Gustaf Samelius, Snow Leopard Trust, Seattle, Washington 98103, USA)

- a. I hypothesize that the previously uncharacterized species has been consistently misidentified as *T. nativa* on the multiplex PCR on multiple occasions
 - b. I hypothesize that the undescribed species will have a broad host and geographic range
 - c. I hypothesize that the undescribed species will be closely related to species/genotypes already established in the Canadian North
 - d. I hypothesize that the undescribed species will be freeze tolerant
4. To compare tissues and serological tests for the detection of *Toxoplasma gondii* in the wolverines
- a. I hypothesize that prevalence and intensity of *T. gondii* will vary between heart and brain
 - b. I hypothesize that performance of specimens (heart fluid vs filter elute) and serological tests (ELISA, IFAT and MAT) to detect antibodies to *T. gondii* will vary between heart and brain
5. To measure the prevalence and intensity of *Toxoplasma gondii* in the wolverines
- a. I hypothesize that prevalence and intensity will be high in wolverine in northwestern Canada, based on their apex position in the food web and scavenging lifestyle
 - b. I hypothesize that age (adult than juvenile wolverines), sex (male than females), and body condition index (higher than lower BCI), will be the risk factors for the *T. gondii* infection and intensity in wolverines

TRANSITION STATEMENT CHAPTER 2

To determine prevalence, larval burden, and genetic diversity of *Trichinella* in wolverines, I first had to determine tissue predilection sites to be targeted for sampling. For *Trichinella*, sites are well defined in some domestic animals, such as horses (*Equus caballus*) and pigs (*Sus scrofa*), and usually include tongue, diaphragm and masseter muscle [126]. No information on muscle predilection sites of *Trichinella* spp. in large carnivores, such as wolverines, is available. This study was thus conducted to compare larval intensity of tongues and diaphragms of wolverines, which were the two tissues for which we had archived samples.

CHAPTER 2 : TONGUE HAS HIGHER LARVAL BURDEN OF *TRICHINELLA* SPP. THAN DIAPHRAGM IN WOLVERINES (*GULO GULO*)

Rajnish Sharma¹, N. Jane Harms², Piia M. Kukka², Sarah E. Parker³, Alvin A. Gajadhar¹, Thomas S. Jung², Emily J. Jenkins¹

¹Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, Canada

²Department of Environment, Government of Yukon, 10 Burns Road, Whitehorse, Yukon, Y1A 2C6, Canada

³Centre for Applied Epidemiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, Canada

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2.1 Abstract

Trichinella is an important zoonotic parasite found in a range of wildlife species harvested for food and fur in Canada. We compared larval intensity from tongue and diaphragm, the best predilection sites in other animal species, from naturally infected, wild wolverines (*Gulo gulo*) (n = 95). Muscle larvae of *Trichinella* spp. were recovered by the pepsin/HCl artificial digestion method (gold standard) using double separatory funnels, and species were identified using multiplex PCR. Prevalence was 83% (79/95). Of those positive for *Trichinella* spp. (n = 79), 76 (96.2%) were detected in both tissues, 2 (2.5%) were positive only on diaphragm, and 1 (1.3%) only on tongue. A total of 62 of 79 wolverines (78.5%) had higher larval burden in tongue than in diaphragm, whereas 17 wolverines (21.5%) had higher larval burden in diaphragm. The predilection site (higher larval burden) of *Trichinella* spp. larvae did not vary significantly between juvenile and adult wolverines (P = 0.2), between male and female wolverines (P = 0.9), and among wolverines classified as having low and high larval intensities overall (P = 0.2). *Trichinella* T6 was the predominant genotype (63 of 79; 80%), followed by *T. nativa* (T2) (6 of 79; 8%). Mixed infections of T2 and T6 were observed in 9 of 79 (12%) wolverines. Larval intensity of *Trichinella* T6 was higher in tongues than diaphragms. No statement can be made for T2 due to insufficient T2 positive samples. In conclusion, tongues are a better site for sampling than diaphragms in future surveys of *Trichinella* larval intensity in wolverines; however, either tissue is suitable for prevalence studies.

2.2 Introduction

Trichinella is an important parasite in wildlife harvested for food and fur in Canada. *Trichinella spiralis*, *T. nativa*, *T. britovi*, *T. murelli*, *T. nelsoni*, and *Trichinella* genotypes T6, T8 and T9 are included in the en-capsulated clade whereas *T. pseudospiralis*, *T. papuae*, and *T. zimbabwensis* constitute the non-encapsulated clade [10]. Five of species/genotypes of *Trichinella* (*T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murelli*, and *Trichinella* T6) are known to occur in domestic or wild animals in Canada [16]. *Trichinella nativa* and *Trichinella* T6 are the most common genotypes in Canadian wildlife [127]. Both produce freeze resistant larvae and are important from a public health point of view, especially in northern Canada, where communities eat game meat raw, frozen, or after pre-paring it by traditional methods such as smoking, drying and fermentation [128]. Outbreaks of human trichinellosis have been reported after consumption of meat from wildlife [129, 130]. It is therefore important to monitor prevalence, intensity of infection, and species of *Trichinella* present in wildlife in Canada. Though most wild carnivores are not consumed, they are important indicators of *Trichinella* and thus can act as target host species in surveillance programs [131]. *Trichinella* infection has been reported in a variety of carnivorous hosts in Canada [15, 17], with the highest prevalence of *Trichinella* infection observed in wolverines (*Gulo gulo*, 77%) followed by polar bears (*Ursus maritimus*, 66%) and cougars (*Felis concolor*, 44%; [16]. Due to their top position in the food web as well as their scavenging behavior [132], large carnivores may be more exposed and thus excellent indicator species of the presence of this nematode.

Methods to detect *Trichinella* in animals include serological tests (e.g., ELISA, Western blot), trichinascopy, and artificial digestion methods [11]. Serological methods targeting antibodies indicate only exposure to *Trichinella*, whereas trichinascopy and the artificial digestion method (DSDM) are assays of direct detection of the parasite. Trichinascopy, however, is largely considered an obsolete technique due to low sensitivity, and the artificial digestion method is the recommended method for detection and recovery of *Trichinella* larvae in muscles of infected animals [11]. Despite higher sensitivity of the digestion method, for surveys of wild carnivores, where mean larval intensity (measured as larvae per gram, LPG) can be low, large sample volumes are often needed.[101] As well as sample size, selection of sampling sites in the host is also a key factor [11]. Predilection sites for *Trichinella* larvae are those muscles that consistently contain the most larvae in infected animals and are often the only sites with detectable larvae in lightly infected animals [16]. Predilection sites for *Trichinella* spp. larvae depend on host species, parasite species

and age [133, 134]. These sites are well de-fined in some domestic animals, such as horses (*Equus caballus*) and pigs (*Sus scrofa*), and usually include tongue, diaphragm and masseter muscle [134]. Information on muscle predilection sites of *Trichinella* spp. is available for some species of large carnivores (e.g. foxes [*Vulpes* spp.], raccoon dogs [*Nyctereutes procyonides*]) but not for wolverines. Such knowledge would be helpful for determining the preferred sampling site for epidemiological surveillance of *Trichinella* infection in wolverines. The primary objective of this study was to compare tongues versus diaphragms from wolverines to determine the better predilection site for this species. The secondary objective was to find if host age, sex, and infection intensity are associated with the predilection site for *Trichinella* spp. larvae in wolverines.

2.3 Methodology

2.3.1 Sample collection

Fur trappers were solicited to voluntarily submit wolverine carcasses during the 2012/2013 and 2013/2014 winter seasons in Yukon, Canada, where wolverines are legally harvested. Skinned carcasses (n = 95), were kept frozen at -20 °C for 6–10 months prior to necropsy. For each wolverine carcass sex was determined, a premolar extracted for aging, and tongues and diaphragms collected for *Trichinella* testing. Age was determined via cementum analysis at a commercial laboratory (Matson's Laboratory LLC, Milltown, Montana, USA). Wolverines < 2 years old were classified as sub-adults and those ≥ 2 years old as adults [135, 136]

2.3.2 Double separatory funnel digestion method

Trichinella first stage larvae (L1) were recovered by the pepsin/HCl artificial digestion assay using the double separatory funnel digestion method [127]. Briefly, tissues from each animal were thawed and connective tissue and fat were removed. Ten grams of each muscle were homogenized in a blender, digested in 1% Pepsin-HCl solution at 37 °C for 60–90 min, followed by sieving through a 180-micron mesh sieve and then sequential sedimentation through two separatory funnels. Digestion was considered complete if less than 0.3 g tissue was left. Solution (20 ml) was drained from the second funnel into a gridded Petri dish and examined under a stereo-microscope for the presence of larvae. *Trichinella* larvae were counted and larva per gram (LPG) of each positive sample was calculated by dividing total number of larvae by weight of digested tissue. Average LPG for each wolverine was calculated from tongue and diaphragm LPG [(LPG of tongue + LPG of diaphragm)/2]. The mean of this average for all positive wolverines was used as a cut-off LPG for categorizing wolverines into wolverines with relative low and high larval intensity.

To identify *Trichinella* species, larvae were collected in 1X PCR buffer and stored at -20°C until processing for DNA extraction [102].

2.3.3 Multiplex polymerase chain reaction

For *Trichinella* species identification, DNA from five samples consisting of an individual larva and a sixth sample consisting of 10 pooled larvae per positive animal were analyzed by a standard multiplex PCR assay (http://www.trichinellosis.org/uploads/PCR_Guidelines.pdf; [102]. Six reference species obtained from the Canadian Food Inspection Agency's Centre for Foodborne and Animal Parasitology were used as controls (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murreli* and *Trichinella* T6).

2.3.4 Statistical analysis

Since tongues and diaphragms originate from the same animal, the proportion of positive results were compared with techniques for matched samples. McNemar Chi-square tests were performed to compare the proportion of wolverine positive for the presence of *Trichinella* from tongues and diaphragms. The kappa value (k) was calculated to determine the agreement between detection using tongues and diaphragms. Kappa values of ≤ 0.40 , $0.40-0.60$, $0.61-0.80$ and ≥ 0.81 were considered to represent slight to poor, moderate to good, substantial and excellent agreement, respectively [137]. Larval burden was defined as the measured LPG. As the dependent variable (LPG) was not normally distributed, we used the Wilcoxon signed rank test (suitable for paired data) to compare mean LPG of tongues and diaphragms. The chi-square test was performed to evaluate significance of the association between predilection site and age, sex and parasitic burden. All statistical tests were performed using commercial statistical software (IBM SPSS, ver. 24; Armonk, New York, USA).

2.4 Results

A total of 79 of 95 (83%) matched samples of tongues and diaphragms were positive for *Trichinella* spp. larvae in at least one tissue. *Trichinella* larvae were recovered from 76 of 79 (96.2%) positive wolverines in both tissues, 2 (2.5%) in diaphragms only, and 1 (1.3%) in tongues only. Sixteen wolverines were negative for *Trichinella* spp. in both tissues. For positive wolverines, proportion of positive tongues (97%, 77/79) and diaphragms (99%, 78/79) was not significantly different ($P = 1.0$). There was excellent agreement between the detection of *Trichinella* larvae from tongues and diaphragms ($k = 0.895$).

Larval burden in tongues ($n = 77$) varied from 0.2 to 166.8 LPG (median = 9.6, 25–75 percentiles = 3.7–38.1), and from 0.1 to 119.3 (median = 5.0, 25–75 percentiles = 0.8–11.6) in

diaphragms ($n = 78$). Of the 79 positive animals, 62 wolverines had higher LPG in tongues than in diaphragm, whereas 17 had higher LPG in diaphragm. Tongues had significantly ($P \leq 0.001$, Wilcoxon Signed Rank test, $n = 79$) higher median larval intensity (8.50 LPG) than diaphragms (4.90 LPG).

The cut-off for classifying wolverines into two categories (low and high larval burden) was 18.37 LPG. Wolverines with an average LPG between 0.01–18.37 LPG and > 18.37 LPG were classified as animals with low and high larval burden, respectively. Within each of the different age groups (adults and sub-adults), sexes (males and females) and larval burden categories, tongues had significantly higher LPG than diaphragms Table 2-1. We found no significant differences in site with higher LPG between subadult and adults ($P = 0.2$), male and female ($P = 0.9$), or those with low and high larval intensity ($P = 0.2$; Table 2-2).

Table 2-1 Comparisons of median larvae per gram (LPG) of *Trichinella* spp. between age-groups (adults vs. subadults), sexes, and parasitic burden (low vs. high), in tongues and diaphragms of free-ranging wolverines (*Gulo gulo*; n = 79).

Parameter		n	Predilection Site		Significance Level (P value) ^b
			Median Tongue LPG (5%–95%)	Median Diaphragm LPG (5%–95%)	
Age ^c	Adults	34	9.0 (0.4–94.3)	3.7 (0.1–77.9)	< 0.001
	Subadults	45	8.0 (0.1–86.5)	5.1 (0.2–70.2)	< 0.001
Sex	Males	52	12.4 (0.1–86.0)	5.6 (0.2–70.1)	< 0.001
	Females	27	8.0 (0.7–127.5)	4.0 (0.1–79.7)	< 0.001
Parasitic Burden ^d	Low	54	5.6 (0.2–24.3)	2 (0.1–11.2)	< 0.001
	High	25	49.7 (14.2–152.7)	21.8 (0.7–116.3)	< 0.001

^aPercentiles.

^b Test statistics are from Wilcoxon sign rank test for matched samples. All with 1 df.

^c Wolverines < 2 years old were classified as sub-adults and those ≥2 years old as adults.

^d Wolverines with an average LPG between 0.01–18.37 LPG and > 18.37 LPG were classified as animals with low and high larval burden, respectively.

Table 2-2 Comparisons of sites of highest LPG between age-groups (adults vs. subadults), sexes, and parasitic burden (low vs. high), in tongues and diaphragms of free-ranging wolverine (*Gulo gulo*; n = 79).

Parameter		n	Predilection Site		Test Statistic (P value) ^a
			Tongue	Diaphragm	
Age ^b	Adults	34	29	5	0.2
	Subadults	45	33	12	
Sex	Males	52	41	11	0.9
Parasitic Burden ^c	Females	27	21	6	
	Low	54	40	14	0.2
	High	25	22	3	

^aTest statistics are from McNemar's chi-square test for matched samples. All with 1 df.

^b Wolverines < 2 years old were classified as sub-adults and those ≥2 years old as adults.

^c Wolverines with an average LPG between 0.01–18.37 LPG and > 18.37 LPG were classified as animals with relative low and high larval burden, respectively.

Trichinella T6 was the predominant genotype (63 of 79, 80%), followed by *T. nativa* (T2; 8%) in wolverines from our samples. Mixed infections of T6 and T2 were observed in 9 of 79 (12%) wolverines. For one animal, it was not possible to determine genotype of recovered larvae. Wolverine tongues had a significantly higher larval intensity (LPG) of *Trichinella* T6 than diaphragms (Median LPG 7.4 vs 3.1, $n = 63$, $P = 0.001$). No statement can be made for T2 due to insufficient T2 positive samples.

2.5 Discussion

The selection of appropriate sampling sites plays a vital role in surveillance programs for foodborne parasites such as *Trichinella*. We compared two muscle types (tongue and diaphragm) from wolverines naturally infected with *Trichinella* spp. using the artificial digestion method with double separatory funnels. Higher infection levels were associated with tongues rather than diaphragms, irrespective of host age, sex, or larval burden.

A higher burden of *Trichinella* larvae in tongues versus diaphragms of naturally infected wolverines is comparable with that reported in experimentally infected hosts such as raccoon dogs; [138], foxes; [134, 139, 140], and pigs [98]. In contrast, diaphragms were reported to have higher parasite burden than tongues in naturally infected arctic foxes (*Vulpes lagopus*) from Greenland, although the highest larval burden was in the muscles of eyes and legs [139], which could be due to differences in host species. Other studies also suggest that muscles of the limbs may have a high intensity of *Trichinella* larvae [134, 138, 139]. In experimentally infected raccoon dogs and naturally as well experimentally infected foxes [138-140], leg muscles were the best predilection sites for *Trichinella* larvae. This might be a reflection of these species using their leg muscles to a greater degree in searching, digging and hunting their prey. Our observation of high *Trichinella* larval intensity in tongues and diaphragms of wolverines is likely a result of high activity levels of these muscles, as larval burden depends on the motorial potential and blood flow of a particular muscle [133, 141]. Further work is needed to determine if leg muscles are another potential predilection site for *Trichinella* in wolverines.

Various factors such as larval burden and age of infection as well as of host can influence distribution of *Trichinella* larvae in a host species [133, 134, 139]. Similar to our findings, no significant relationship was established between the distribution of larvae in muscles and age of host in species such as pigs, foxes and horses [134, 139]. We observed higher larval burden in tongues than diaphragms in both infection level groups (wolverines with low and high larval

intensity). In naturally infected wolverines, the parasitic burden may have been a reflection of the initial infectious dose as previous studies have shown positive correlation between the inoculation dose and parasitic burden [142].

Our finding that tongues had more LPG of *Trichinella* T6 than diaphragm is consistent with observations that distribution of muscle larvae is influenced more by the species of host rather than the species of *Trichinella* [134]. Evidence of a comparable muscle distribution of different *Trichinella* species has been observed in various hosts, including foxes and raccoons [134, 139, 140, 143]. For example, similar predilection sites were reported in arctic foxes [139, 140] and raccoon dogs [138] infected with *T. spiralis* and *T. nativa*. One limitation of our study is that the small sample size of positive wolverines infected with *T. nativa* did not allow for any meaningful statistical evaluation to compare tongues and diaphragms for this genotype. Therefore, further studies are required to determine the distribution of *T. nativa* in muscles of wolverines. In addition, there is a need to determine the distribution of *Trichinella* spp. larvae in different muscles.

2.6 Conclusion

In conclusion, tongues are a better predilection site than diaphragms for *Trichinella* spp. larvae in naturally infected wolverine, irrespective of the age, sex and parasite burden. In comparison to diaphragm, the tongue is easier to collect, and does not require opening the abdominal or pleural cavity of the carcass, which can be challenging in field conditions. However, there may be occasions when other muscles are preferred; for example, in carnivore species or populations in which rabies is endemic, collecting tongues could place the sample collector at risk of exposure to infective saliva. Our study suggests that either tissue is a good sample for testing for trichinellosis in wolverine if prevalence (rather than larval intensity) is the desired goal. Further work is needed to determine if other muscles (e.g leg, eye, or other muscles.) could act as better sample site for detecting this parasite in wolverines.

2.7 Acknowledgements

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TRANSITION STATEMENT CHAPTER 3

From the previous chapter, I concluded that tongue is a better predilection site than diaphragm for *Trichinella* spp. larvae in naturally infected wolverine, irrespective of the age, sex and parasite burden. In comparison to diaphragm, the tongue is easier to collect, and does not require opening the abdominal or pleural cavity of the carcass, which can be challenging in field conditions. Thus, I decided to collect tongues to determine status of *Trichinella* infection in wolverines. In this chapter, I determined prevalence and larval intensity of *Trichinella* in wolverines. I also analysed association of *Trichinella* prevalence and intensity with risk factors including age, sex, location etc. In addition, I determined the genotypes of *Trichinella* circulating in wolverines of Yukon.

CHAPTER 3 : PREVALENCE, INTENSITY, AND GENETIC DIVERSITY OF *TRICHINELLA* SPP. IN WOLVERINE (*GULO GULO*) FROM NORTHWESTERN CANADA

Rajnish Sharma^{1*}, N. Jane Harms², Piia M. Kukka², Thomas S. Jung^{2,3}, Sarah E. Parker⁴, Sasha Ross¹, Eric P. Hoberg⁵ and Emily J. Jenkins¹

¹ Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, Canada

² Department of Environment, Government of Yukon, P.O. Box 2703, Whitehorse, Yukon, Y1A 2C6, Canada

³ Department of Renewable Resources, University of Alberta, 351 General Services Building, Edmonton, Alberta, T6H 3T1, Canada

⁴ Centre for Applied Epidemiology, Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, S7N 5B4, Canada

⁵ Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, New Mexico, 87131-0001, U.S.A.

Author contributions

Conceived and designed the experiments: RS, EJJ, NJH, SEP; Collection of samples: RS, NJH, PAK, TSJ; Provided mentorship, logistics and supplies: NJH, PAK, TSJ, SEP, EPH, EJJ; Performed the experiments: RS, SR; Analyzed the data: RS, SEP; Wrote the manuscript: RS, EJJ; Approved the final version of the manuscript: RS, NJH, PAK, TSJ, SEP, SR, EPH, EJJ.

*Corresponding author: ras863@mail.usask.ca

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3.1 Abstract

Species of *Trichinella* are globally important foodborne parasites infecting a number of domestic and wild vertebrates, including humans. Free-ranging carnivores can act as sentinel species for detection of *Trichinella* spp. Knowledge of the epidemiology can help prevent *Trichinella* spp. infections in northern Canadian animals and people. Previous research on *Trichinella* spp. in wildlife from the Yukon did not involve geographically extensive sampling with large sample sizes, nor did it identify risk factors associated with *Trichinella* spp. infection, or the species of *Trichinella*. In a cross sectional study, we determined the prevalence, infection intensity, risk factors, and species or genotypes of *Trichinella* in wolverine (*Gulo gulo*) harvested for fur in two regions of the Yukon from 2013–2017. Using a double separatory funnel digestion method, we found larvae of *Trichinella* in the tongue of 78% (95% CI = 73–82) of 338 wolverine sampled. The odds of adult (≥ 2 years) and yearling (1–2 years) wolverine being *Trichinella* spp.-positive were 4 and 2 times higher, respectively, compared to juveniles (< 1 year). The odds of *Trichinella* spp. presence was 3 times higher in wolverine from southeast than northwest Yukon. The mean intensity of infection was 22.6 ± 39 (SD, range 0.1–295) larvae per gram of muscle tissue. Using multiplex PCR, *Trichinella* T6 was the predominant genotype (76%), followed by *T. nativa* (8%); mixed infections with *Trichinella* T6 and *T. nativa* (12%) were observed. As well, *T. spiralis* was discovered in one wolverine, which possibly indicates overlap of sylvatic and domestic life cycles. Due to their scavenging habits, apex position in the food chain and wide home range, wolverine hosted high prevalence, larval intensity and multiple species of *Trichinella*. Therefore, wolverine (especially adult males) should be considered as sentinel species for surveys for *Trichinella* spp. across their distributional range.

3.2 Introduction

Trichinella spp. are among the most significant foodborne parasites listed by the World Health Organization/United Nations Food and Agriculture Organization [3]. All *Trichinella* species and genotypes have been reported in mammals, while *T. pseudospiralis* also develops in avian hosts, and *T. papuae* and *T. zimbabwensis* have also been reported from reptilian hosts. All species and genotypes of *Trichinella* are zoonotic and are transmitted by consuming raw or improperly cooked meat [144].

Globally, most human outbreaks of trichinellosis have been attributed to domestic pigs infected with *T. spiralis* [4]. However, almost all human outbreaks in the last 20 years in Canada involved *T. nativa* and *Trichinella* T6 linked to consumption of raw or improperly cooked game meat, commonly from walrus (*Odobenus rosmarus*) and bears (*Ursus* spp.) [85-88]. For example, 15 trichinellosis incidents have occurred in Nunavik from 1982-2009; of these, nine were linked to walrus meat [88]. One of the largest outbreaks reported in Canada was from Saskatchewan, where 78 people became sick after consuming black bear (*Ursus americanus*) meat infected with *T. nativa* [86]. A recent outbreak of human trichinellosis due to *T. nativa* in Ontario was associated with consumption of dried meat of black bear and resulted in rare thrombotic complications [87]. *Trichinella* T6 has also been identified in an outbreak in Ohio, USA, linked to consumption of bear meat from Canada [85]. Knowledge of epidemiology of the disease and pathways for exposure can help prevent *Trichinella* spp. infections in northern Canadian animals and people.

A recent survey in Canada conducted over a period of 10 years on 15 wildlife species showed an overall *Trichinella* spp. prevalence of 20% in tested animals [16], and identified *T. nativa*, *Trichinella* T6, *T. murrelli* and *T. pseudospiralis* [16].

Free-ranging carnivores are considered reservoir hosts of *Trichinella* spp., and can act as sentinel species for detection of *Trichinella* spp. For example, various species of canids have been previously proposed as sentinel species for *Trichinella* spp. [70-72]. Adequate availability (relative population stability), measurable response (e.g. parasites in tissues, antibodies in blood), earlier response than people or sympatric wild species (high chance of exposure early in life and a relatively short life span), and high levels of exposure are the significant characteristics of an ideal sentinel animal host [75, 76]. Wolverine (*Gulo gulo*) may also be an ideal sentinel species for detecting *Trichinella* spp. in northwestern North America because: (1) they are legally killed for their fur and carcasses are available from fur harvesters [145]; (2) initial studies have reported

relatively high prevalence and intensity of *Trichinella* spp. infection in wolverine, suggesting that they may be a key species in the epidemiology of *Trichinella* spp. [16, 67]; and (3) they are a facultative scavenger [132, 146, 147] near the top of terrestrial food webs, and as such may be bio-accumulators of foodborne parasites such as *Trichinella* spp.

To the best of our knowledge, there are three published studies on species of *Trichinella* infecting wild carnivores from the Yukon [16, 23, 25]. The two older studies (45 years ago) reported *Trichinella* spp. in 71% of 21 grizzly bear (*Ursus arctos*) from the Yukon [25] and 47% of 127 gray wolves (*Canis lupus*) from the Yukon and Northwest Territories [23]. Larvae of *Trichinella* spp. were detected in Arctic fox, wolf and wolverine from the Yukon in a more recent survey [16]. Previous research on *Trichinella* in wildlife from the Yukon did not involve geographically extensive and site intensive sampling, or large sample sizes. Secondly, researchers did not study associated risk factors and identify the species of *Trichinella* larvae isolated. To fill these knowledge gaps, we sampled a large number of wolverine from across the Yukon.

Our objectives were: (1) to determine the prevalence and intensity of *Trichinella* spp.; (2) to explore the association between potential risk factors and *Trichinella* spp.-positivity and larval intensity in wolverine; and (3) to definitively identify the species/genotypes of *Trichinella* and their distribution in the Yukon.

3.3 Materials and methods

3.3.1 Wolverine sampling

We obtained wolverine carcasses from across Yukon, Canada, which is ~483 000 km² in area and sparsely populated by people (~0.08 people/km²), with 76% of the human population living in the City of Whitehorse [148]. The topography of the Yukon is rugged and characterised by mountain ranges, plateaus, and valleys and lowlands, with much of the land area being remote. Climate is subarctic continental, with annual precipitation ranging from 250–600 mm, most of which falls as snow from October to May. The mean daily temperature ranges from -15°C to -30°C in January and from 10°C to 15°C in July. The Yukon is characterised by boreal forest in valley bottoms, and shrub communities and alpine tundra at and above the treeline, respectively [149].

Wolverine are legally harvested throughout the Yukon by fur trappers during the winter [148, 150, 151], with an average of 132 ± 31 (SD) animals harvested annually [148]. Wolverine carcasses were submitted by fur trappers to the government wildlife agency, and kept frozen at -20°C for 6–10 months prior to necropsy. The location and sex of each carcass were recorded, and

age was determined by cementum analysis of premolar tooth at a commercial laboratory (Matson's Laboratory LLC, Milltown, Montana, USA). Wolverine were classified as juveniles (<1 year), yearlings (1–2 years), or adults (≥ 2 year) based on cementum-derived ages. A body condition index (BCI) for each wolverine was calculated using predetermined regression equations for wolverine from our study area that incorporated the mass of select fat depots with body size and sex [151]—higher BCIs corresponded to greater mass-specific fat levels, for each sex, than lower BCIs. Tongues were collected in plastic bags and stored at -20°C until further analyses. Location where each wolverine was harvested was assigned to one of two geographic areas (northwest Yukon and southeast Yukon; **Figure 3.1**), because these geographic areas corresponded with broad phylogeographic divisions for wolverine [152]. We reasoned that phylogeographic divisions could influence the genetic diversity of wolverine parasites [153].

The number of wolverine in our study area was unknown, but estimated to range between 3500–4000 [49]. We used OpenEpi ver. 3.01 [154] to estimate the sample size necessary to be representative of the population of wolverine in the Yukon. We parameterized OpenEpi by using the mid-point of the population size estimate range (3750), and an expected prevalence of *Trichinella* spp. of 80%, based on earlier reports of 88% and 77% [16, 67] in wolverine from northern Canada. Based on these values, the calculated sample size necessary for our study was 231 with a confidence interval of 95%; we obtained 338 wolverine carcasses from animals harvested during 2013–2017, which exceeded the estimated sufficient sample size for our study.

Because we obtained samples from animals that were harvested for purposes other than our research, this was considered exempt from animal care committee review at the University of Saskatchewan. We obtained a wildlife research permit and appropriate export permits from the Government of Yukon.

3.3.2 Recovery of *Trichinella* spp. larvae

As tongue is a known predilection site for *Trichinella* spp. in wolverine [155], tongues were thawed at room temperature and cut into 0.5–1.0 cm cubes, which were mixed and a portion randomly selected to make up to 10 g. Connective tissue and fat were removed and ≤ 10 g of muscle was digested using the double separatory funnel digestion method [127]. Briefly, each sample was homogenized in a blender, digested in 1% Pepsin-HCl solution at 37°C for 60–90 min, followed by sieving through a 180-micron metal mesh sieve and then sequential sedimentation through two separatory funnels. Digestion was considered complete if <0.3 g tissue remained. Solution (20 ml) was drained from the second funnel into a gridded Petri dish and examined under a stereo-

microscope for the presence of larvae. Based on morphology, coiled and ‘C’ (comma) shaped larvae were classified as live and dead, respectively. Larvae per gram (LPG) of each positive sample was calculated by dividing total number of larvae by weight of digested tissue. To identify *Trichinella* species, larvae were collected in 1X PCR buffer and stored at -20°C until DNA extraction [102].

3.3.3 Molecular identification of *Trichinella* spp.

From each wolverine positive for *Trichinella* spp., parasite genomic DNA was extracted from 5 individual larvae as well as a pool of 10 larvae using a Proteinase K extraction method [102, 156]. Briefly, larvae placed in 10X PCR buffer were preheated at 90°C for 10 min and then cooled on ice for 5 minutes. Proteinase K (2 μl) was added and tubes were kept at 48°C overnight. After incubation, each sample was heated at 90°C for 10 min. Samples were then centrifuged at $10000 \times g$ for 5 min and stored at -20°C until further analysis.

To identify species or genotypes of *Trichinella* larvae, primers amplifying internal transcribed spacer regions (ITS 1 and 2) as well as the expansion segment V of the large subunit ribosomal DNA were used in a multiplex PCR assay as previously described [102, 156]. PCR reactions were performed with master mix, containing 1X AmpliTaq Gold 360 Master mix (Applied Biosystems, CA, USA), 50 μM primers, 2 μl of GC Enhancer, 2.5 μl of DNA and ultrapure water to a total volume of 25 μl . The thermocycler (Biorad, USA) conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 90 s, and final elongation at 72°C for 7 min. Species were identified based on the band pattern developed on 2.5% agarose gel when analyzed under UV light using the Gel Doc system (Alpha Innotech AlphaImager digital imaging system). DNA from six species of *Trichinella* (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6) were used as positive controls. Positive controls, negative template control and DNA extraction control (only 10X PCR buffer) were also included in each PCR.

3.3.4 Data analysis

A wolverine was considered positive for *Trichinella* spp. if ≥ 1 larva was recovered from tissue samples. Prevalence with 95% confidence intervals (CI) was calculated from the proportion of wolverine that were positive for *Trichinella* spp. The intensity of infection was measured as LPG, with “low” and “high” intensity defined as ≤ 10 LPG and >10 LPG, respectively. We built binary logistic regression models to independently test for (1) presence of *Trichinella* spp. (present or absent), and (2) intensity of *Trichinella* spp. infection (low or high). We evaluated the following

potential predictors for inclusion in a final multivariable model using univariable logistic regression analysis with a relaxed level of significance ($p \leq 0.20$): Age (juvenile, yearling and adult), sex (female and male), harvest location (southeast Yukon and northwest Yukon), harvest season (4 annual seasons), and body condition index (continuous variable). Weight of muscle tissue processed (< 5 g, 5.0–9.9 g, and ≥ 10 g) was also included as a potential predictor in both models. For our intensity model, *Trichinella* spp. identified (*Trichinella* T6, *T. nativa* and mixed infection) was also included as a predictor. Stepwise forward multivariable logistic regressions were performed to build the final model. Goodness of fit of the final model was evaluated by the Hosmer-Lemeshow test. Variables with a significance level of $p < 0.05$ were retained in the model. To estimate the degree of the association between each predictor and *Trichinella* status, odds ratios and respective 95% confidence intervals were calculated. Median larval intensity in wolverine with *Trichinella* T6 alone and *T. nativa* alone (no mixed infection) was compared by Mann Whitney *U*-test. All statistical analyses were performed using IBM SPSS (ver. 24; Armonk, New York, USA).

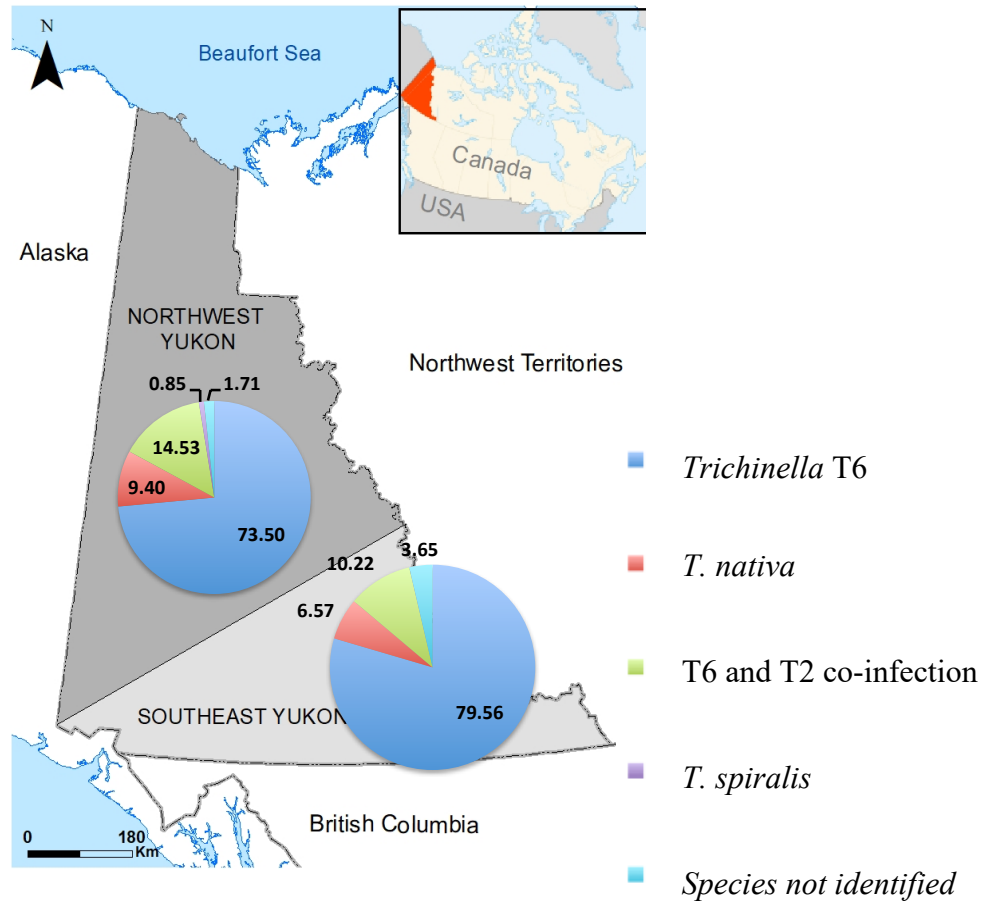


Figure 3-1 Prevalence of *Trichinella* spp. identified in wolverines from two harvest locations of the Yukon

3.4 Results

3.4.1 Sample collection

The mean age and BCI of sampled wolverine were 1.6 years (SD 2.05 range, <1-10) and 8.88 (SD 5.24, range <1-30), respectively. Age was not estimated for 3 of 338 wolverine (0.88%), and harvest location was not available for 12 of 338 wolverine (3.5%). Most carcasses submitted were male [148], with a male to female ratio of 2:1. The age-class distribution of wolverine we sampled was similar among juveniles (37%), yearlings (29%), and adults (34%). Wolverine were obtained in relatively equal percentages (23–27%) from each of the four years of sampling as well as from the two phylogeographic regions; 50% and 46% from the northwest and southeast Yukon, respectively.

3.4.2 *Trichinella* spp. prevalence

Trichinella spp. larvae were detected in 78% of wolverine tested (78%, 95% CI 73–82%; Table 3-1). Larvae of *Trichinella* spp. were detected most frequently in adult wolverine (87%, 99/114 animals sampled, 95% CI= 79–92), followed by yearlings (82%, 79/96, 95% CI= 73–89) and juveniles (65%, 81/125, 95% CI=56–73). More males (80%, 95% CI =74–84) than females (73%, 95% CI= 64–81) were positive for *Trichinella* spp.

Our univariable logistic regressions revealed that age ($p < 0.001$), sex ($p = 0.184$), and phylogeographic region ($p < 0.001$) were significantly associated with the presence of *Trichinella* spp. in wolverine. Our stepwise multivariable regression suggested that age and phylogeographic region were significantly associated with *Trichinella* spp. prevalence (Table 3-2). The odds of *Trichinella* spp. presence were 2 times (odds ratio = 2.25, 95% CI =1.16–4.36; $p = 0.016$) and 4 times (odds ratio = 3.76, 95% CI =1.86–7.60; $p = 0.001$) higher in yearlings and adults, respectively, than in juveniles (<1 yr). The odds of presence of *Trichinella* spp. was 3 times (odds ratio = 2.87, 95% CI =1.60–5.16; $p = 0.001$) higher in wolverine from southeast Yukon than northwest Yukon.

Table 3-1 Prevalence (% positive) of *Trichinella* spp. in 338 wolverine (*Gulo gulo*) in the Yukon

		Number of Positive wolverine	Total animals examined	Prevalence	95% Confidence Interval
Age	Juvenile	81	125	65	56 -73
	Yearling	79	96	82	74 -89
	Adult	99	114	87	79 -92
Sex	Female	82	112	73	64 -81
	Male	180	226	80	74 -84
Harvest location	Northwest	117	169	69	62 -76
	Southeast	137	157	87	81 -92
Harvest year	2013-2014	69	86	80	71 -87
	2014-2015	66	91	73	63 -81
	2015-2016	60	77	78	68 -86
	2016-2017	67	84	80	70 -87
Weight of tongue processed	Less than 5 g	24	36	67	50 -80
	5 -9.9 g	22	28	79	61 -90
	10 g	216	274	79	74 -83

3.4.3 Intensity of *Trichinella* spp. infection

The median intensity of *Trichinella* spp. larvae was 8.4 (range 0.1–295) LPG. Larval intensity >1 LPG, >10 LPG, > 50 LPG and >100 LPG was observed in 89% (232/262 animals sampled), 46% (120/262), 11% (29/262) and 3% (8/262) wolverine, respectively. The median larval intensity of *Trichinella* spp. was higher in juveniles (14 LPG) than in yearlings (7 LPG) and adult wolverine (7 LPG). Age and *Trichinella* species were significantly associated with high larval intensity in both univariable and stepwise multivariable regression (Table 3-2). The odds of high larval intensity were 2 times (odds ratio = 2.46, 95% CI =1.29-4.66; $p = 0.006$) higher in yearlings than adult wolverine. The odds of high larval intensity were 3 times (odds ratio = 3.29, 95% CI =1.03-10.5; $p = 0.044$) higher in wolverine infected with mixed *T. nativa* and T6 infections than *T. nativa*-only infected wolverine (Table 3-2).

Table 3-2 Risk factors for *Trichinella* prevalence and intensity in wolverine (*Gulo gulo*) identified in final stepwise logistic regression, odd ratios (OR), and 95% confidence intervals and probability (p)

Model (outcome) [n]	Predictors in the final model	OR	95% CI	p
<i>Trichinella</i> spp. infection (yes/no) [323]	Age (Juvenile)			0.001
	Age (Yearling)	2.3	1.2-4.4	0.016
	Age (Adult)	3.8	1.9-7.6	0.001
	Harvest Location (Northwest harvest)			0.001
	Southeast Location	2.9	1.6-5.2	0.001
<i>Trichinella</i> spp. larval burden (high/low) [254] ^a	Age (Adult)			0.012
	Age (Yearling)	2.5	1.3-4.7	0.006
	Age (Juvenile)	1.1	0.6-2.1	0.715
	<i>Trichinella</i> spp. (<i>T. nativa</i>)			0.006
	<i>Trichinella</i> T6	0.9	0.3-2.2	0.731
	<i>Trichinella</i> T6 and <i>T. nativa</i>	3.3	1.0-10.5	0.044

n=Animals with complete record for respective model

a= Only positive samples with *Trichinella* species were included in the model; Positive isolates with no species identified or *T. spiralis* identified were excluded

3.4.4 Genetic diversity of *Trichinella* spp.

Trichinella spp. larvae from 254 of 262 (97%) infected wolverine were successfully identified to the species/genotype level. Overall, 85% (222/262) of infected wolverine were infected with a single species, either *Trichinella* T6 or *T. nativa*. *Trichinella* T6 was the predominant genotype (76%), followed by *T. nativa* alone (8%); 12% (32/262) had mixed infections with both *Trichinella* T6 and *T. nativa*. Larvae of *T. spiralis* were present in one wolverine. Prevalence and larval intensity of different species of *Trichinella* identified in wolverine in the Yukon is shown in Table 3-3. Median larval intensity between *Trichinella* T6 alone and *T. nativa* alone was not significantly different ($p = 0.694$, Mann Whitney U test). Within each phylogeographic region sampled, *Trichinella* T6 was the predominant species with prevalence higher than 70% (Figure 3.1). In contrast to *Trichinella* T6, which was more prevalent in wolverine from southeast region, prevalence of *T. nativa* and mixed infections was higher in wolverine from the northwest region (Figure 3.1).

Table 3-3 Prevalence and intensity of different species of *Trichinella* identified in wolverine (*Gulo gulo*) in the Yukon.

Trichinella spp.	Number positive	Prevalence (% 95% CI) n=338	% of Trichinella positive (95% CI), n=262	Median	Range LPG
<i>Trichinella</i> T6 only	199	58.8 (53.6-64.0)	76.0 (70.4-80.7)	7.4	0.1-294.6
<i>T. nativa</i> only	22	6.5 (4.3-9.6)	8.4 (5.6-12.4)	7.6	0.44-134.6
<i>Trichinella</i> T6 and <i>T. nativa</i>	32	9.5 (6.8-13.1)	12.2 (8.8-16.7)	12.5	1.3-146.6
<i>T. spiralis</i>	1	0.3 (0.001-1.7)	0.4 (0.001-2.1)	NA	
<i>Trichinella</i> T6*	231	68.3 (63.2-73.1)	88.2 (83.7-91.5)	8.5	0.1-294.6
<i>T. nativa</i> **	54	16.0 (12.5-20.3)	20.6 (16.2-25.9)	11.5	0.44-146.6
Species identified	254	75.2 (70.3-79.5)	96.9 (94.1-98.4)	8.2	0.1-294.6
Species- not identified	8	2.4 (1.2-4.6)	3.1 (1.6-5.9)	16.9	0.2-33.0

* Includes both *Trichinella* T6 only and mixed infections (*T. nativa* and *Trichinella* T6)

** Includes both *T. nativa* only and mixed infections (*T. nativa* and *Trichinella* T6)

NA= Not applicable

3.5 Discussion

We report high prevalence (78%) of *Trichinella* spp. (predominantly T6) in wolverine of the Yukon, Canada, based on the largest sampling effort yet for this species ($n = 338$ wolverine). Prevalence of *Trichinella* spp. appears to be substantially higher in wolverine than all other carnivores tested so far in northern Canada or Alaska, with the possible exception of grizzly bears (*Ursus arctos*; Table 3-4). Prevalence of *Trichinella* spp. in wolverine in our study was comparable to other reports from northern Canada, where prevalence of 77% and 88% had been noted [16, 62, 67], and higher than that observed from this species in other countries (0% in Sweden, 30% in Russia, 50% in the USA) [64-66] (Table 3-4). Variation in study prevalences may be due to differences in sample size, type of muscle sampled (tongue, diaphragm, cheek muscles, or leg muscles), or methodology (trichinoscopy versus muscle digestion) [62, 64, 157, 158]. In the current study, we used the pepsin-HCl digestion method, which has higher sensitivity as compared to trichinoscopy and compression [62, 64, 158]. Digestion is now considered the gold standard for detection of larval *Trichinella* spp. in animal tissues [11]. We used tongue tissue, which has been demonstrated to be a better sampling site than diaphragm for detection of larval *Trichinella* spp. in wolverine [155]. Finally, we used relatively large samples of tongue tissue for examination (89% of samples were ≥ 5 g), as recommended for studies of wild carnivores [101].

Our data indicates a positive association between *Trichinella* spp. infection (prevalence) and increasing age, as demonstrated for other wildlife species in Europe [159, 160] but not in a similar study on wolverine in Nunavut [67]. The reason for higher prevalence of *Trichinella* spp. infection in older animals likely reflects that they have had more time to encounter prey infected with the parasite than younger age classes. Higher prevalence of *Trichinella* spp. in male than female wolverine was observed, consistent with findings from a similar survey in Northwest Territories, Canada [24], whereas almost equal prevalence rates (89% in male vs 86% in female wolverine) were reported in wolverine from Nunavut, Canada [67]. Males likely consume more food and have larger home ranges than females [55], increasing chance of exposure, and male biases in parasitism are fairly common across many genera.

Table 3-4 Prevalence of *Trichinella* spp. in wild carnivores in Canada and Alaska

Host	Location	%	Total number tested	Genotype (Number of isolates of <i>Trichinella</i> spp. genotyped)	Reference
Arctic fox (<i>Vulpus lagopus</i>)	NT	3	1566	ND	[22]
	NU, NT	11	28	ND	[16]
	AK	10	117	ND	[66]
Black bear (<i>Ursus americanus</i>)	NT	6	120	T2 (4), T6 (2), ND (1)	[161]
	QC	1	107	ND	[162]
	BC, SK, QC, NT	7	193	BC- T2 (NM), SK-T2 (NM), QC-T2 (NM), NT-T2 (NM) & T6 (NM)	[16]
	AK	24	21	ND	[66]
	AK	28	40	ND	[163]
Bob cat (<i>Lynx rufus</i>)	NS	4	24	ND	[16]
Coyote (<i>Canis latrans</i>)	NS, BC	1	346	ND	[16]
	AK	13	8	ND	[66]

Ermine (<i>Mustela erminea</i>)	AK	43	40	ND	[66]
	AK	9	11	ND	[66]
Fisher (<i>Martes pennant</i>)	BC, NS	29	17	ND	[16]
Grizzly Bear (<i>Ursus arctos</i>)	YT, NT	86	24	ND	[25]
	NT	73	11	T6 (6), T2 & T6 (1)	[161]
	BC, NU	29	68	T6 (NM)	[16]
	AK	50	20	ND	[66]
Lynx (<i>Lynx canadensis</i>)	BC, NU	7	107	T6 (NM)	[16]
	AK	24	17	ND	[66]
Marten (<i>Martes americana</i>)	BC, NU	3	101	ND	[16]
North American Cougar (<i>Puma concolor cougar</i>)	BC	44	127	BC (mainland) T2 (NM), T6 (NM), BC (Island) T4 (NM), T5 (NM)	[16]
Polar bear (<i>Ursus maritimus</i>)	QC, NU	66	85	T2	[16]
	AK	53	17	ND	[66]
	AK	47	478	ND	[163]
Red Fox (<i>Vulpes vulpes</i>)	NT	11	19	ND	[22]

	AB	6	18	ND	[22]
	AK	41	76	ND	[66]
Skunk (<i>Mephitis mephitis</i>)	AB	6	124	ND	[16]
Wolf (<i>Canis lupus</i>)	NT	13	8	ND	[22]
	YT, NT	47	153	ND	[23]
	NT	52	27	T2 (6), T6 (5)	[161]
	AB	33	3	ND	[22]
	QC	50	2	ND	[22]
	NL	4	48	ND	[22]
	YT, BC, NU	43	28	T2	[16]
	AK	36	148	ND	[164]
	AK	33	154	ND	[66]

ND= Not done, NM= number not mentioned, AB=Alberta, AK=Alaska, BC=British Columbia, MB=Manitoba, NS=Nova Scotia, NT= Northwest Territories, NU=Nunavut, QC=Quebec, Saskatchewan= SK, YT=Yukon.

Trichinella spp. were widely distributed in wolverine across the Yukon; however, prevalence was higher in the southeast region than in the northwest region. There may be regional variation in wolverine diet, which may play a role in prevalence differences among regions. Other factors that can affect regional differences in prevalence of *Trichinella* spp. may include environmental factors such as snow cover, altitude, air temperature or humidity [165], or anthropogenic factors. Previously, the number of snow cover days was positively associated with the incidence of *Trichinella* spp. infections among wild boars from Latvia [165]. However, based on this assumption alone, a greater number of snow cover days in the northern Yukon suggest that prevalence should have been higher in the northern regions.

In our study we observed a median infection intensity (8 LPG) that was twice as high as that reported from an earlier study in northwestern North America (3.7 LPG) [16] (Table 3-5). In contrast with the age-related prevalence differences, we observed higher median larval intensity in juveniles than yearlings and adults. This suggests that while risk of exposure increases with age, the severity of infection was higher following exposure in young animals, which could be due to juveniles having less immunity [166].

Table 3-5 Prevalence and larval burden (larvae per gram) of *Trichinella* spp. in wolverine from different geographical locations

Geographical Location	No. tested	No. (%) positive	Method (specimen)-muscle weight	Mean larval burden (Range), Median^s	Genotypes	Reference
North America						
YT	347	271(78)	MD (T, D)-10	22.1 (0.1-295), 8 ^s	T2, T6, T1	Present study
BC	4	2 (50)	MD/COMP (T, D, O)-NM	NM	ND	[157]
MB	1	NA	NM	NM	ND	[167]
NT	38	0 (0)	COMP	NA	NA	[62]
NT	49	12 (25)	MD (T)		ND	[24]
NU	41	36 (88)	MD (D)-5	8.6 (0.2-51.8)	T2, T6	[67]
NT*, BC, YT	111	85 (77)	MD (T, D, O)-10	20.8 (0.025-522), 3.7 ^s	*T2, T6	[16]
AK	38	19 (50)	MD (NM)-NM	3.5 (0.2-18)	ND	[66]
Iowa	1	1 (100)	MD-BT (T, D, O)	NM (10)	ND	[168]
Europe						
Sweden	27	0(0)	MD/COMP (D, O)-NM	NA	ND	[64]
Asia						
Kamchatka (Eastern Russia)		(30)				[65]
Chukotka Peninsula Jakucja	1	1	MD (NM)-NM	NM	T2	[169]

Jakutia	1	1	NM	NM	T1	[170]
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AK=Alaska, BC=British Columbia, BT= Baermann technique, COMP= Compressorium, MB= Manitoba, MD=Muscle digestion, NT= Northwest Territories, NU= Nunavut, NM=Not mentioned, ND= Not done, T2= *T. nativa*, T6= *Trichinella* T6, YT=Yukon, T=Tongue, D=Diaphragm, O= muscle other than diaphragm and tongue, NA- Not applicable

Our study is the first to determine the genotype and/or species of *Trichinella* circulating among wildlife from the Yukon, including two sylvatic *Trichinella* species (*Trichinella* T6 and *T. nativa*), and, unexpectedly, a domestic species (*T. spiralis*) (discussed in detail in Appendix A). Due to their freeze tolerance, larvae of both *T. nativa* and *Trichinella* T6 are expected in arctic and sub-arctic regions. However, *T. spiralis* is not freeze tolerant and the significance of this finding is uncertain. We report higher prevalence of *Trichinella* T6 than *T. nativa* in wolverine from the Yukon, consistent with observations elsewhere in the western Canadian Arctic (Nunavut). *Trichinella* T6 is apparently the most common genotype in Canadian wildlife, with wider host and geographic ranges than *T. nativa* [16]. Differences in infectivity among genotype or species of *Trichinella* warrant further study.

Our results show that *Trichinella* T6 and *T. nativa* occur in sympatry geographically (Yukon) and in the same individual host (wolverine). Co-infection with *T. nativa* and *Trichinella* T6 has been previously reported in wolverine [67] and hybridization between the 2 species has been observed in Alaska [171]. The multiplex PCR (targets nuclear DNA only) does not distinguish hybrids of *Trichinella* T6 and *T. nativa*, which requires microsatellite and/or mitochondrial DNA analysis [172]. Based on a study in wolverine from Nunavut, microsatellite and mitochondrial DNA analysis showed that hybrids appear as *T. nativa* on the multiplex PCR, and that 2 *Trichinella* isolates identified as T6 on the multiplex were actually *T. nativa* and one was half T6 and half *T. nativa*. These observations are consistent with cross-hybridization, but rarely introgression, between *Trichinella* T6 and *T. nativa* in wolverine from Nunavut, based on T6 isolates from a relatively small geographical area [172]. Future work should include microsatellite analysis on both T6 and *T. nativa* isolates from different geographical locations from the Yukon and more broadly across North America to determine if *Trichinella* T6 can be considered as a distinct species.

3.6 Conclusion

Wolverine, especially adult males, are likely good sentinels for *Trichinella* spp. in northwestern North America largely because they have a high prevalence, intensity, and diversity of *Trichinella* spp.; wolverine can host at least 3 species of *Trichinella*. Moreover, as a species that is highly sought by fur trappers across large spatial scales, there is the potential for a ready supply of samples to monitor for change in *Trichinella* prevalence, intensity, or diversity. As such, we suggest the use of wolverine for monitoring changes in *Trichinella* spp. infection in wildlife of

northwestern Canada. Larval intensity greater than or equal to 1 LPG is considered a significant risk for food safety [16], and most (89%) wolverine positive for *Trichinella* spp. in our study met or exceeded this threshold. Although wolverine are not regularly consumed as food by people, proper personal protective practices (i.e. wearing gloves and thoroughly washing hands before eating, and storing wolverine carcasses away from other meat intended as food) should be practiced by fur trappers handling wolverine carcasses to prevent transmission. Practices such as leaving carcasses in the field, or using wolverine meat as bait for trapping or hunting, may also facilitate transmission, even in winter as larvae of sylvatic species of *Trichinella* survive freezing.

3.7 Acknowledgements

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TRANSITION STATEMENT CHAPTER 4

In the previous chapter, I found high prevalence, larval intensity, and genetic diversity of *Trichinella* spp. in wolverines in the Yukon. Therefore, they can be considered as good sentinels for *Trichinella* spp. in northwestern Canada. *Trichinella* T6 was the predominant genotype of *Trichinella*, followed by *T. nativa*; mixed infections were also present. My results show that *Trichinella* T6 and *T. nativa* occur in sympatry in wolverines from the Yukon. These two genotypes remain genetically discrete in zones of contact or in sympatry, and consequently T6 may merit species status. To explore this, I decided to pursue further studies on deeper genetic characterization of *Trichinella* T6 and *T. nativa*. While confirming genotyping results derived from a commonly used multiplex PCR by DNA sequencing, I discovered a previously unrecognized lineage representing a putative cryptic species of *Trichinella* in a wolverine from the Canadian North. This lineage could not be distinguished from and was identical to *T. nativa* based on currently applied multiplex PCR. In this chapter, I will outline the phylogeny, geography and host range, and biological characteristics (encapsulation, freeze tolerance) of this previously unrecognized species.

CHAPTER 4 : A PREVIOUSLY UNRECOGNIZED CRYPTIC SPECIES OF *TRICHINELLA* IN WOLVERINE FROM THE CANADIAN NORTH

Rajnish Sharma^{1*#}, Peter Thompson^{2*}, Eric P Hoberg³, Brad Scandrett⁴, Kelly Konesci⁴, N Jane Harms⁵, Piia M Kukka⁵, Tom S Jung⁵, Brett Elkin⁶, Robert Mulders⁶, N C Larter⁷, Marsha Branigan⁸, Jodie Pongracz⁸, Brent Wagner¹, Benjamin Rosenthal^{2**} and Emily Jenkins^{1**}

¹ Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

² USDA, Agricultural Research Service, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705, USA.

³ Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131-0001

⁴ Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada.

⁵ Environment, Yukon Government, P.O. Box 2703, 10 Burns Road, Whitehorse, YT, Canada, Y1A 2C6.

⁶ Environment and Natural Resources, Government of the Northwest Territories, 600, 5102-50th Avenue, Yellowknife, NT, Canada, X1A 3S8

⁷ Government of the Northwest Territories, Department of Environment and Natural Resources, PO Box 240, Fort Simpson, Northwest Territories X0E 0N0, Canada.

⁸ Environment and Natural Resources, Government of the Northwest Territories, P.O. Box 2749, Shell Lake, Inuvik, NT, Canada, X0E 0T0

Conceived and designed the experiments: RS, PT, EPH, BS, RB, EJJ; Provided mentorship, logistics and supplies: PT, EPH, BS, KK, NJH, PMK, TSJ, BE, RM, NCL, MB, JP, BW, RB, EJJ; Collected samples and supplied specimens: RS, BS, KK, NJH, PMK, TSJ, BE, RM, NCL, MB, JP. Performed the experiments: RS, PT, BW; Analyzed the data: RS, PT, EPH, RB, EJJ; Wrote the manuscript: RS, PT; Approved the final version of the manuscript: RS, PT, EPH, BS, KK, NJH, PMK, TSJ, BE, RM, NCL, MB, JP, BW, RB, EJJ.

* First co-authors; ** Senior co-authors.

#Corresponding author ras863@mail.usask.ca

Status of manuscript: In preparation

4.1 Abstract

Trichinellosis is caused by nematodes of the genus *Trichinella*, which has two major clades. The encapsulated clade includes six species and three genotypes, whereas the non-encapsulated clade includes three species. In North America including Canada, five species of *Trichinella* (*T. spiralis*-T1, *T. nativa*- T2, *T. pseudospiralis*- T4, *T. murrelli*-T5 and *Trichinella* T6) have been found in homeothermic vertebrate hosts. Here we discuss the discovery, phylogeny, geography and host range of a previously unrecognized cryptic species of *Trichinella* designated as the T13 genotype. Phylogenetic analysis showed that a lineage containing T1 + *T. nelsoni*- T7 was the sister group for T13 and the remaining species in the subclade of encapsulated *Trichinella* (i.e *T. patagoniensis*-T12, T2, T6, *T. britovi*-T3, T8, T5, T9). A relatively basal position for T13 was unexpected and indicates a historically deep divergence, pre-dating the later radiation of an assemblage of species distributed across the Holarctic, Nearctic and Neotropical regions. Of 95 animals tested using PCR-RFLP, 68 were hosts for *Trichinella nativa* based on the mitochondrial genome of muscle larvae, 2 were hosts for *Trichinella* T6, and 11 had mixed infections of *T. nativa* and *Trichinella* T6. Specimens of a putative cryptic species of *Trichinella* were found only in wolverines, and 14 animals were infected either as single infection (11/14), or as mixed infections with *T. nativa* and *Trichinella* T6. Specimens designated as T13 occur in sympatry with *Trichinella* T6 and *T. nativa* geographically (Yukon, Northwest Territories) and in the same host (wolverine), although T13 is not closely related to T2 or T6. Multiplex PCR used in the standard identification of *Trichinella* isolates has misidentified this putative cryptic species, as *T. nativa*. We recommend use of a newly developed PCR-RFLP or sequencing to confirm identification of the isolates of *T. nativa* or any species of *Trichinella*, respectively. Our results indicate that T13 is not geographically widespread in Canada, and may be limited to wolverine in Northwestern Canada, especially the Yukon, suggesting a possible historical link to Beringia and the Palearctic. Exploration of Alaskan and Siberian isolates may contribute to further resolution of a geographically complex history for *Trichinella* and other parasites across the Western Hemisphere, Beringia, and Eurasia.

4.2 Introduction

Trichinellosis, an important parasitic zoonosis globally, is caused by nematodes of the genus *Trichinella* [11]. The only source of transmission of *Trichinella* spp. is by consuming meat of infected animals [11]. Among 24 significant food borne parasites listed by the World Health Organization/United Nations Food and Agriculture Organization, *T. spiralis* ranks 7th in public health significance and 1st in global trade significance, while other *Trichinella* spp. ranked 16th and 7th, respectively [3]. Outbreaks of trichinellosis have been reported worldwide, and are linked with consuming raw or undercooked meat, primarily from pig, horse, wild boar, bear and walrus.

Trichinella was considered a monotypic genus until 1972 [173]. Thereafter, researchers using different methods (e.g. biology, alloenzymes, crossbreeding, biogeography and molecular markers) started describing new species despite a lack of distinctive morphological features, and an incomplete consideration of species concepts, limits, and criteria. In 1972, three species were added in the genus *Trichinella*, and from 1992 until 2008, eight more species and genotypes were discovered [173-181]. So far, 12 distinct lineages of the genus *Trichinella* have been recognized, with 9 named species and three unique genotypes that have not been named. These species are grouped into encapsulated and non-encapsulated clades based on the presence or absence of the collagen layer surrounding the nurse cell containing the first larval stage (L1) [144, 182].

The encapsulated clade includes six nominal species (*T. spiralis* T1, *T. nativa* T2, *T. britovi* T3, *T. murrelli* T5, *T. nelsoni* T7, *T. patagoniensis* T12) and three unnamed genotypes (*Trichinella* T6, T8, and T9), whereas the non-encapsulated clade includes three species (*T. pseudospiralis* T4, *T. papuae* and *T. zimbabwensis*) [182]. All species and genotypes of *Trichinella* are zoonotic, circulating among mammals; however, *T. pseudospiralis* has also been found in birds and *T. papuae* and *T. zimbabwensis* in reptiles. *Trichinella spiralis* and *T. pseudospiralis* are globally distributed [12, 13], and all other species are relatively restricted geographically. For example, *T. nativa* is restricted to the Holarctic region, with its southern boundary between the isotherms -5 and -4°C in January [14]. Besides geographical distribution, species of *Trichinella* vary in other characteristics including capacity to infect host animal species, and control measures (freeze resistant vs. freeze susceptible).

In North America including Canada, five species of *Trichinella* (*T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6) have been found in terrestrial vertebrates [15, 16]. In Canada, *T. spiralis* has been eradicated from commercially raised pigs and is now rarely reported in backyard pigs [16-20]. The latest Canada wide survey involving multiple wild animal

species did not find *T. spiralis* [16]; however, it has been reported in coyotes (*Canis latrans*) and red foxes (*Vulpus vulpus*) from Prince Edward Island in the late 1990s [21], and in a wolverine originating from the Yukon (Appendix A). *Trichinella pseudospiralis* and *T. murrelli* have been reported rarely in wild animals from Canada. *Trichinella pseudospiralis* has been reported only twice in wild animals from Canada; the first case was from a Mountain lion (*Puma concolor*) originating from Vancouver Island in 2003 [16], and a second case was from a wolverine collected from the South Slave region, Northwest Territories (Appendix B). The only documented report of *T. murrelli* in Canada was from North American cougar (*Puma concolor cougar*) [16]. *Trichinella nativa* and *Trichinella* T6 are the most common species in free-ranging mammals from Canada, with prevalence varying from 1-77 % among several carnivores [15, 16]. These species of *Trichinella* are sympatric in terms of geography and within individual hosts, including wolves [171] and wolverines [67].

In a recent survey, we surveyed 338 wolverines for *Trichinella* infection and identified species/genotypes using a multiplex PCR [102]. While confirming multiplex genotyping by DNA sequencing, we unexpectedly discovered an undescribed cryptic species of *Trichinella* in a wolverine from the Canadian North. Specimens of this undescribed species appeared identical to *T. nativa* in multiplex PCR, which is considered the standard for species-level identification within the genus. This led to a broader survey of purported *T. nativa* larvae in a range of carnivore hosts across the Canadian North using newly developed methods with sufficient discriminatory power to recognize species diversity among congeners within *Trichinella*. Herein, we describe larval morphology and explore phylogeny, geography and host range necessary to characterize and propose new taxonomy for a newly recognized cryptic species of *Trichinella*.

4.3 Material and methods

4.3.1 Discovery of a cryptic species

4.3.1.1 Recovery and identification of *Trichinella* spp. larvae

Larvae of *Trichinella* species were recovered from previously frozen muscle tissue by the double separatory funnel digestion method [127]. DNA was extracted from pools of 15 larvae of *Trichinella* spp. from an individual host using Promega DNA IQ kits (Promega, Madison, WI), as per manufacturer's instructions. Multiplex PCR was performed to identify the species of larvae as per earlier protocol [102].

4.3.1.2 Whole genome sequencing

Amount of DNA was measured by a Qubit fluorometer (ThermoFisher, Massachusetts, USA), as per manufacturer instructions. Whole genome sequencing (WGS) was performed on the Illumina MiSeq platform (Illumina Inc., San Diego, CA). Libraries were prepared using Nextera DNA Flex library kits (Illumina Inc., San Diego, CA) as per manufacturer instructions. Briefly, DNA was fragmented by topoisomerase and linked to Illumina specific adapters (see manufacturer specifications). Labeled fragments were amplified by PCR with the following conditions: 98°C for 3 min, 6 cycles of 98°C, 40 sec; 62°C, 30 sec; 68°C 2 min followed by 68°C, 2 min. Final libraries were obtained by cleaning the amplified DNA with magnetic beads (Beckman Coulter Life Sciences, Indiana, USA). Libraries were quantified on Qubit flurometer, and diluted to 8 pico molar and run on Illumina MiSeq. Initial library quality was determined by running on a MiSeq v2 nano kit and libraries were then submitted to a full paired-end 300 bp MiSeq V3 run. Reads obtained were exported in FASTQ format, and data was analyzed using Geneious ®11.1.5.

4.3.1.3 Data analysis

4.3.1.3.1 Denovo Assembly

Paired-end reads were trimmed to have no bases lower than Q20 (<1 in 100 chance of an error) and no more than 2 bases with Q-scores lower than Q30 (<1 in 1000 chance of an error) prior to assembly. We performed de novo assembly of the WGS reads using Geneious Assembler with Med-low sensitivity and Genious v.11.1.5. The mitochondrial genome was identified based on BLAST searches of contigs against complete mitochondrial genomes of 12 species of *Trichinella* downloaded from GenBank [183]. Reads were also mapped to chromosomal reference of *T. spiralis* [184] using standard Geneious assembler (Sensitivity: medium low / Fast) to reach consensus.

4.3.1.3.2 Annotation

Annotations were transferred from mitochondrial genomes of *T. nativa* (N 025752) [183] based on homology, and listed chromosomes of *T. spiralis* [184] to the mitochondrial genome and chromosomes of the new species of *Trichinella*, respectively.

4.3.1.3.3 Phylogenetic analysis

Phylogenetic trees were generated for both mitochondrial and nuclear DNA sequences for all *Trichinella* genotypes. For comparison of nuclear genomes, fifteen single copy orthologs (SCO) present in all *Trichinella* species/genotypes were identified [185]. Single copy orthologs were extracted from 15 loci, concatenated for each species/genotype, and aligned using MAFFT.

Mitochondrial sequences were aligned separately as they have a different mode of inheritance. JModel Test was used to determine the best substitution model for tree building. Phylogenetic trees were built using maximum likelihood (PHYML used GTR substitution model), and neighbor-joining algorithms (as implemented in Geneious tree builder with the HKY substitution model) to determine the evolutionary relationships among new and known species/genotypes of *Trichinella*. In order to confirm the relationships in the concatenated nuclear and mitochondrial trees, we also evaluated the relationships of all species/genotypes using five SCOs individually.

4.3.2 Geographical distribution and host range of a cryptic species

4.3.2.1 Isolates of *Trichinella nativa* as classified by multiplex PCR

Larvae or extracted DNA of *Trichinella nativa* were obtained from 95 animals. Genomic DNA was extracted from 5 individual larvae using a Proteinase K extraction method [102, 156]. Larvae were identified as *T. nativa* based on primers amplifying internal transcribed spacer regions (ITS 1 and 2) as well as the expansion segment V of the large subunit ribosomal DNA in a multiplex PCR assay as previously described [102, 156]. Forty-one isolates were from wolverines from Yukon and Northwest Territories (NT), and 44 isolates from other animal host species were provided. A detail of host animal species with their geographical origin is shown in *Table 4-1*.

Table 4-1 *Trichinella nativa* isolates: Animal hosts and their geographic origin

Animal Host	Total number	Geographical origin (number)
Bear	1	BC (1)
Black Bear	1	NT (1)
Cougar	9	BC (8), AB (1)
Coyote	1	QC (1)
Fisher	2	BC (2)
Fox	25	NT (9), QC (16)
Grizzly Bear	1	BC (1)
Polar Bear	3	NT (2), QC (1)
Raccoon	1	QC (1)
Walrus	1	QC (1)
Wolf	8	NL (3), NT (5)
Wolverine	42	NT (27), YT (15)
Total	95	NT (44), QC (20), YT (15), BC(12), NL (3), AB (1)

4.3.2.2 Polymerase chain reaction- Restriction fragment length polymorphism (PCR-RFLP)

To differentiate the cryptic species of *Trichinella* from *T. nativa*, we designed a novel PCR-RFLP assay. The PCR reaction targeted the mitochondrial cytochrome b (Cytb) gene using the primer pair Cytb-JLF199 5'- ATACGAGAAGTAAAATTTGG-3' and Cytb JLR1093- 5'- TCATTGAATAGAGTGTGGTT-3' which were designed using Primer3 [186] and plug-in Geneious ®11.1.5 [187]. Each reaction contained 1X Taq polymerase (Amplitaq Gold 360 master mix, Applied Biosystems, CA), 1 µl of each primer, 3 µl GC enhancer (Applied Biosystems, CA), 3 µl genomic DNA, and molecular grade H₂O to make a final volume of 35 µl. Gradient PCR was performed to optimize hybridization temperature for primer annealing. Finally, PCR was conducted in a thermocycler (Biorad, USA) with the following conditions: 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min, then a final extension step at 72 °C for 10 min. Amplified PCR products of approximately 920 bp were digested using BsaI (New England Biolabs) and BglII (New England Biolabs) in buffer NEB 3.1 (New England Biolabs) at 37°C for 2 hrs. *Trichinella nativa*, *Trichinella* T6 and the new species of *Trichinella* were differentiated based on the band pattern developed on 2% agarose gel under UV light using the Gel Doc system (Alpha Innotech AlphaImager digital imaging system) (Figure 4-1). DNA from 3 species of *Trichinella* (*T. nativa*, *Trichinella* T6, and new species of *Trichinella*) were used as positive controls in each run of multiplex-PCR and PCR-RFLP as well as a no-DNA negative control.

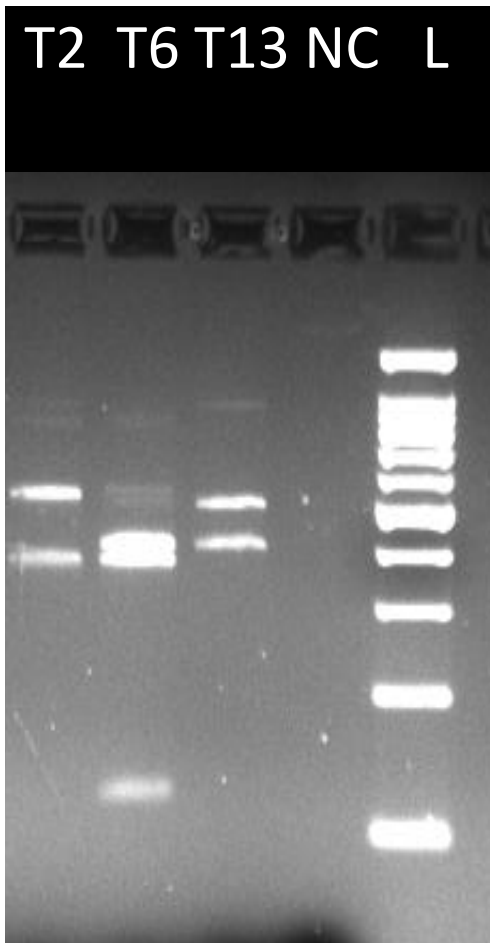


Figure 4-1 Restriction fragment length polymorphism differentiated *T. nativa*, *Trichinella* T6 and previously unrecognized species of *Trichinella* (T13)

T2= *T. nativa*, T6= *Trichinella* T6, T13= previously unrecognized species of *Trichinella*, NC= No template control

4.3.2.3 Motility test and Compressorium

Previously frozen tongue tissues from 14 wolverine positive for the cryptic species were digested using a double separatory funnel digestion [102]. Motility of the larvae from 9 positive wolverine was examined by incubating a Petri dish containing 50 larvae at 37⁰ C for 30 min. The number of motile larvae was counted and expressed as percentage motility. Compression of small muscle samples between glass plates (compressoria) was also performed to determine the encapsulation of the larvae.

4.4 Results

4.4.1 Genome of cryptic species

The draft assembly of the genome was 52,094,442 bp, and GC content was 42.7 %. Scaffold lengths ranged from 997 bp to 711531 bp (mean 11099, SD= 31237). Mapping to chromosomal reference of *T. spiralis* [184] resulted in three chromosomes of the new species of *Trichinella*. The complete circular mitochondrial genome of the new species of *Trichinella* was 14072 bp, and G + C content was 34.4. Similar to other species of *Trichinella*, the mitochondrial genome of new species has 13 protein-coding genes (atp6, atp8, nad1-6, nad4L, cox1-3, cytb), two rRNA genes (rrnS and rrnL) and 22 tRNA genes (size ranged from 53-75 bp).

4.4.2 Phylogenetic relationship

As expected, phylogenetic analysis showed two separate clades: encapsulated and non-encapsulated. The previously unrecognized cryptic species of *Trichinella* clustered in a relative basal position within the encapsulated clade, supported strongly by bootstrap (bs=100) values. Within the encapsulated clade, the putative cryptic species did not group with T1 and T7, and was placed as the basal taxon within a larger subclade as the sister of *T. patagoniensis* and (T2 + T6), (T3 + T8), and (T5 + T9). Phylogenetic trees constructed from both the mitochondrial genome (*Figure 4-2*), and 15 concatenated SCOs (both rooted and un-rooted; *Figure 4-3*, *Figure 4-4*, respectively) showed a similar topology, and a consistent position for the cryptic lineage of *Trichinella*. Similar phylogenetic relationships were demonstrated when trees were built individually for five SCOs (Furin, Epi, Fox, ACVR2A, pcbp3). Phylogenies were consistent in indicating a basal divergence for the cryptic

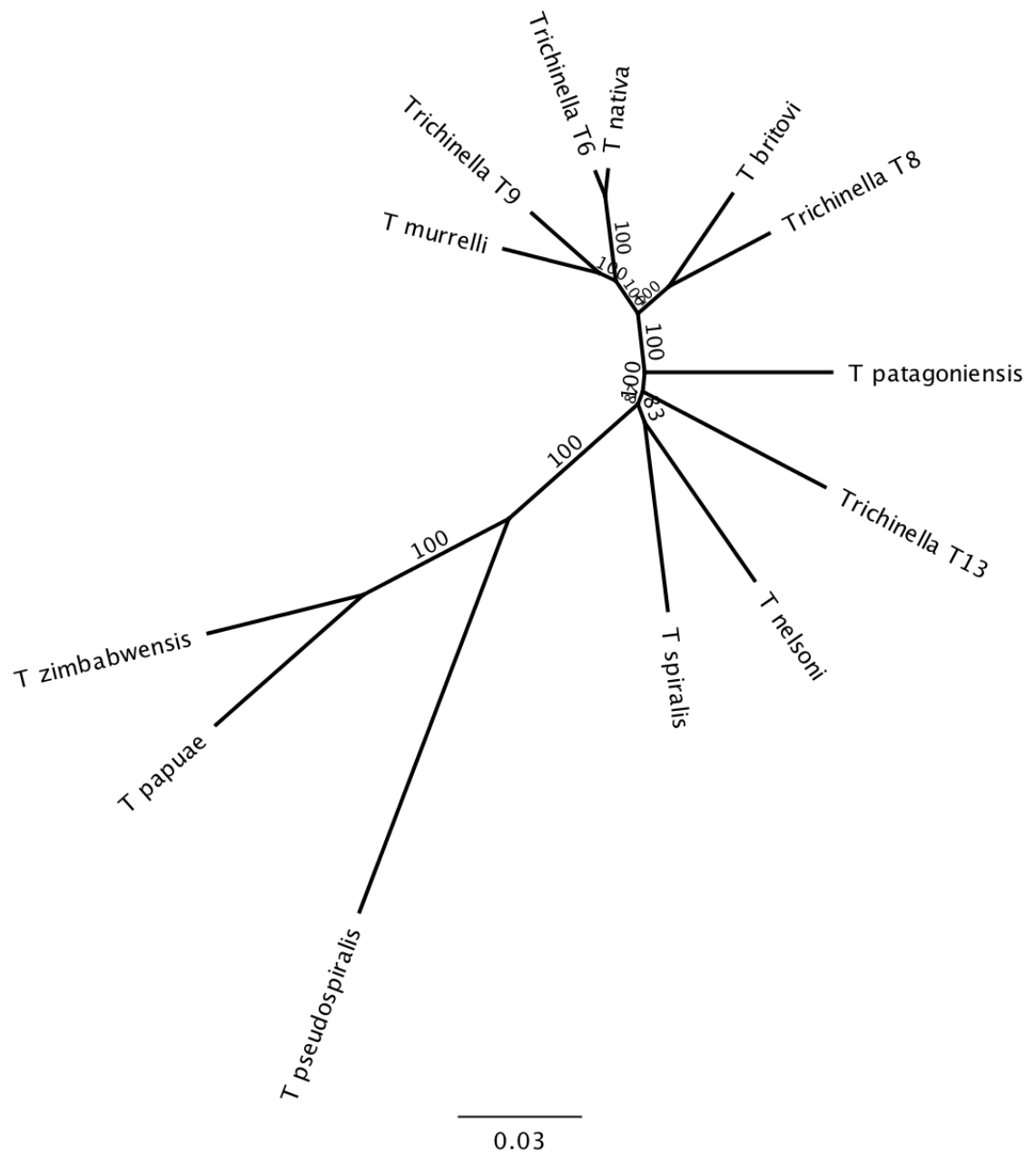


Figure 4-2 Relationships among the described (T1–T12) and an undescribed species of *Trichinella* based on analyses of mitochondrial nucleotide sequences using NJ and Maximum likelihood algorithms

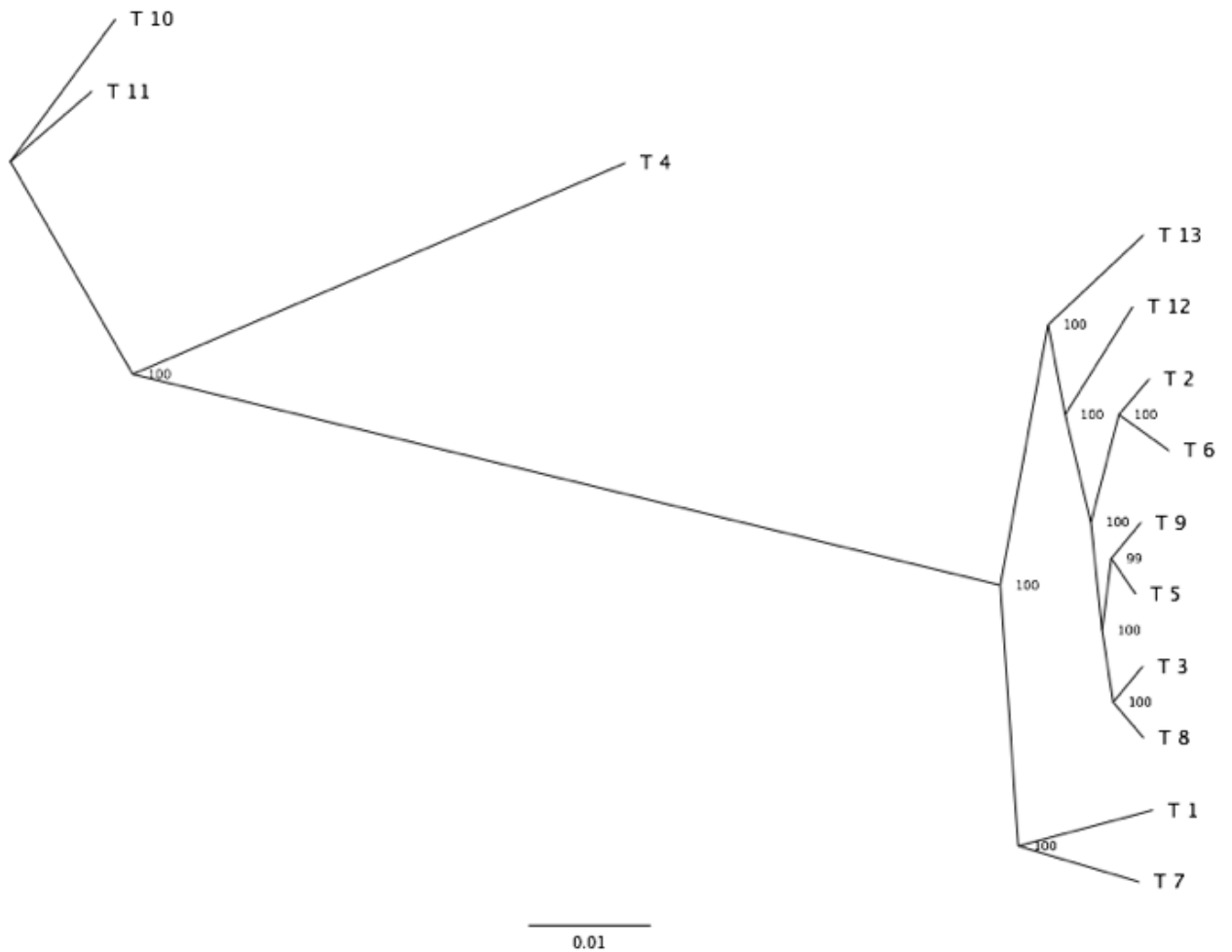


Figure 4-3 Relationships among the described (T1–T12) and cryptic species of *Trichinella* based on analyses of 15 concatenated SCOs’ nucleotide sequences using NJ and Maximum likelihood algorithms (Unrooted)

T1 = *T. spiralis*, T2 = *T. nativa*, T3 = *T. britovi*, T4 = *T. pseudospiralis*, T5 = *T. murrelli*, T6 = *Trichinella* T6, T7 = *T. nelsoni*, T8 = *Trichinella* T8, T9 = *Trichinella* T9, T10 = *T. papuae*, T11 = *T. zimbabwensis*, T12 = *T. patagoniensis*, T13 = cryptic species of *Trichinella*

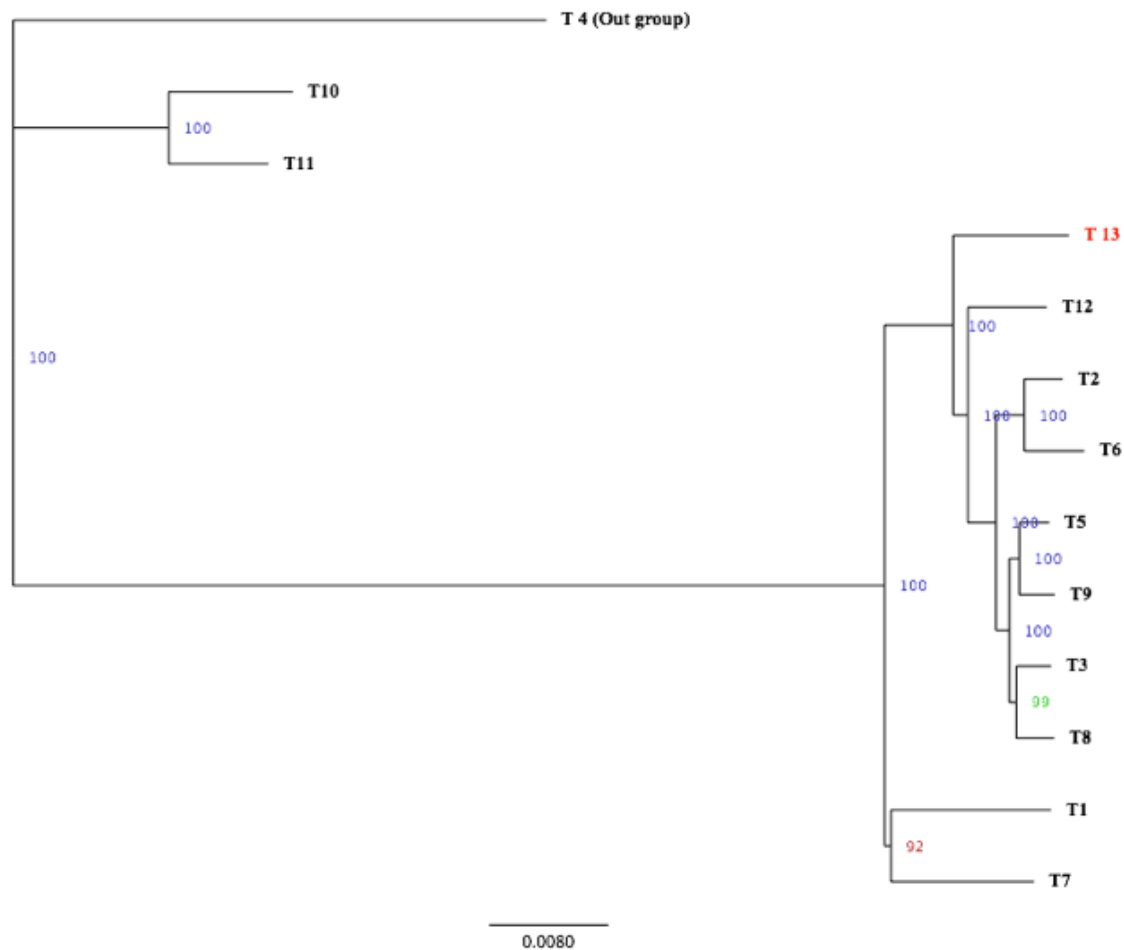


Figure 4-4 Relationships among the described (T1–T12) and cryptic species of *Trichinella* based on analyses of 15 concatenated SCOs nucleotide sequences using NJ algorithms (Rooted) with T4 as an out group

T1 = *T. spiralis*, T2 = *T. nativa*, T3 = *T. britovi*, T4 = *T. pseudospiralis*, T5 = *T. murrelli*, T6 = *Trichinella* T6, T7 = *T. nelsoni*, T8 = *Trichinella* T8, T9 = *Trichinella* T9, T10 = *T. papuae*, T11 = *T. zimbabwensis*, T12 = *T. patagoniensis*, T 13 = cryptic species of *Trichinella*

species as the sister for a larger assemblage of *Trichinella* in Eurasia and the Western Hemisphere (excluding *T. spiralis* and *T. nelsoni*), and that it was not closely related to the geographically sympatric *T. nativa* and T6.

4.4.3 Multiplex PCR and Mitochondrial PCR-RFLP

Out of the 95 isolates examined by multiplex PCR, 90 isolates were purportedly *Trichinella nativa*, and mixed infection with both *T. nativa* and *Trichinella* T6 was detected in wolverine and cougar (Table 4-2). PCR using primers targeting the Cytb gene of mtDNA produced a 920 bp band in *T. nativa*, *Trichinella* T6, and a putative cryptic species of *Trichinella*. On RFLP, DNA of *T. nativa* showed two bands of approx. 537 and 377 bp, and DNA of *Trichinella* T6 showed three bands of approx. 407, 377, and 130 bp. The cryptic species of *Trichinella* showed two bands of approx. 507 and 407 bp (Figure 4-1). Out of 95 animals tested, 68 were *T. nativa* in their mitochondrial genome, 2 were *Trichinella* T6, and 11 had mixed infection of *T. nativa* and *Trichinella* T6. The putative cryptic species of *Trichinella* was found only in wolverines, and 14 animals (out of 42) were infected either as single infection (11/14), or as mixed infections with *T. nativa* and *Trichinella* T6 (Table 4-2). There was a higher number of wolverines positive for the cryptic species from the Yukon versus Northwest Territories (10 vs. 4). Harvest location within each territory and demographics of positives wolverines are shown in Table 4-3.

4.4.4 Motility test and compressorium

Larvae per gram of the new species of *Trichinella* ranged between 1 -135. Almost all positive wolverines had a higher proportion of live (coiled) than dead larvae. Only one motile larva was present in one sample (percent motility 0.03%). Compression showed that specimens of the cryptic species of *Trichinella* are encapsulated (Figure 4-5).

4.5 Taxonomic summary

We discovered a new species of *Trichinella* (designated as T13), for which taxonomic nomenclature will be proposed in publication.

Host: Wolverine (*Gulo gulo*), ID 17-49, 1-year-old male.

Location: This isolate originated from a wolverine harvested in the Old Crow region of Yukon. Old Crow (67°N 139°W) is a community located in the north of the Yukon.

Etymology: To be determined

Table 4-2 Distribution of new species of *Trichinella* among animal hosts

		Designation based on							
		Multiplex PCR		Mitochondrial PCR-RFLP					
Animal Host	Total number	T2	T2 + T6	T2	T6	T2+ 6	T13	T2+ T13	T6+ T13
Bear	1	1	0	1	0	0	0	0	0
Black Bear	1	1	0	0	0	1	0	0	0
Cougar	9	7	2	4	1	4	0	0	0
Coyote	1	1	0	1	0	0	0	0	0
Fisher	2	2	0	2	0	0	0	0	0
Fox	25	25	0	23	0	2	0	0	0
Grizzly Bear	1	1	0	1	0	0	0	0	0
Polar Bear	3	3	0	3	0	0	0	0	0
Raccoon	1	1	0	1	0	0	0	0	0
Walrus	1	1	0	1	0	0	0	0	0
Wolf	8	8	0	8	0	0	0	0	0
Wolverine	42	39	3	23	1	4	11	2	1
Total	95	90	5	68	2	11	11	2	1

Table 4-3 Demographic details of wolverines positive for new species of *Trichinella*

Territory	Harvest Location	ID	Age (yr)	Sex	BCI	Harvest Season
Yukon	Old Crow Flats	14-98	1	Male	4.46	2013-2014
		17-49	1	Male	8.33	2016-2017
	Mackenzie Mountains	15-26	2	Female	10.68	2014-2015
	Yukon Plateau North	15-13	3	Male	6.22	2014-2015
	Yukon Plateau North / Selwyn Mountains	17-14	1	Male	3.36	2016-2017
	Yukon Plateau Central	15-38	6	Female	10.03	2014-2015
	Pelly Mountains	14-43	1	Female	6.98	2013-2014
	Ruby Ranges	14-40	1	Male	4.65	2013-2014
		17-64	7	Male	9.61	2016-2017
	Yukon Southern Lakes	14-26	2	Male	6.99	2013-2014
Northwest Territories	Inuvik	11/26	1	Male	12.5	2011
	Dehcho	W10-112	8	Female	.	2009
		WV11-093	2	Male	16.9	2011
	South Slave	W10-013	9	Male	1.05	2009

YT= Yukon; NT= Northwest Territories; BCI= Body condition Index; ID= Animal ID

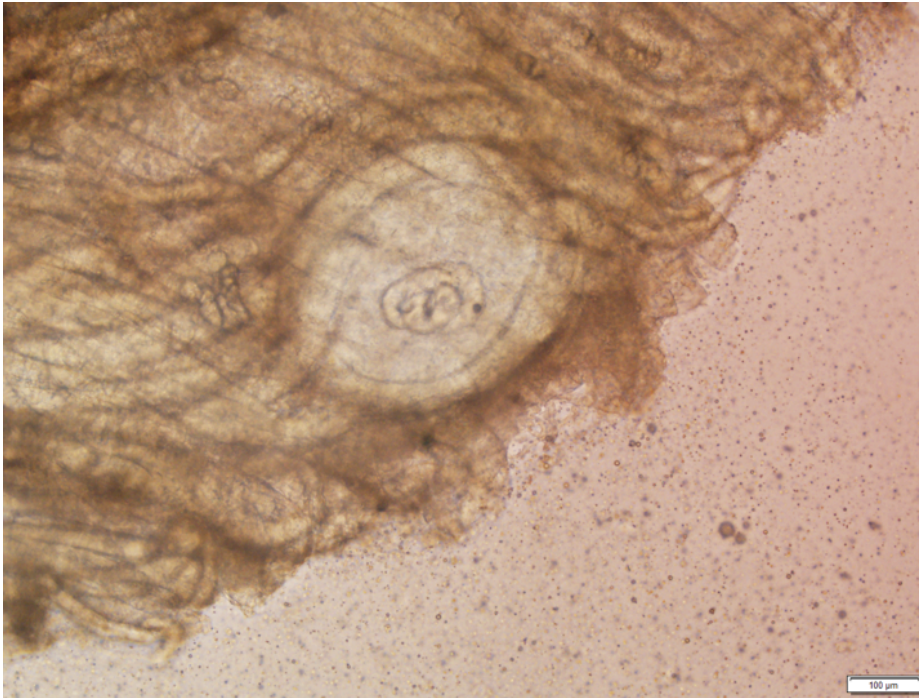


Figure 4-5 Encapsulated larva of the undescribed species of *Trichinella* in the tongue muscle of wolverine (*Gulo gulo*)

4.6 Discussion

We report the discovery of a previously undescribed cryptic species of *Trichinella* (designated as T13) in wolverine from the Yukon. Specimens of this cryptic species were initially identified as *T. nativa* using a common genotyping system considered to be standard in the identification and diagnosis of *Trichinella*. This newly recognized species was not closely related to North American or Eurasian encapsulated species of *Trichinella*, especially T6 and *T. nativa* that occur in sympatry at high latitudes. The genus *Trichinella* now includes 13 species recognized or taxonomically designated in the last 50 years; the most recent nominal taxon, *T. patagoniensis*, was discovered 10 years ago [10, 177, 178, 180, 181]. Context for recognition of a cryptic lineage, the T13 lineage, in wolverine was based on the comparison of the whole mitochondrial genome as well as 15 nuclear single copy orthologs from all the known species of *Trichinella*, rather than the comparison of single gene markers used in earlier discoveries of species or genotypes of *Trichinella*. For example: cytochrome c-oxidase subunit I (COI) was targeted during discovery of *Trichinella* T9 [181]; expansion segment 5 (ES5) for *T. papuae* [177]; COI, ES 5, and mitochondrial-lsrDNA for *T. zimbabwensis* [178]; and cytochrome COI, ES5 and 5S ribosomal DNA intergenic spacer region (5S ISR) for *T. patagoniensis* [180]. We used maximum likelihood and neighbor-joining algorithms to determine the relationship between T13 and currently described species of *Trichinella*. All trees constructed had similar topology and strong support values at the nodes (bs = 92-100 in tree based on SCO, and bs = 83-100 tree based on mitochondrial genome). Additionally, using 5 SCO individually shared by all the species of *Trichinella* as well as T13, phylogenetic trees (data not shown) did not demonstrate any differences in topology, which further strengthened our findings.

Overall, within the encapsulated clade, the lineage which diverged into T1+T7 is the sister of a lineage which contains T13 and a diverse assemblage of Holarctic and Nearctic and Western Hemisphere species of *Trichinella*. Our analysis provides phylogenetic support for T13 within this subclade as the sister of *T. patagoniensis* and the remaining encapsulated species (T2, T6, T3, T8, T5, T9). A deep divergence resulting in *T. patagoniensis* within the encapsulated group, estimated near 6-10 million years ago, has been consistent in all recent analyses [185]. Topology that places T13 as the sister of *T. patagoniensis* and the remaining species is consistent with a deeper age of origin for the former lineage.

Table 4-4 Intensity, motility, morphology and designation of species/genotype based on nuclear loci in the multiplex PCR and PCR-RFLP of the mitochondrial genome of the larvae obtained from wolverines positive for T13.

ID	Multiplex PCR	Mito PCR-RFLP	LPG	L/D Live/Dead	Motility (%)
14-26	T2	13	134.6	133.6	0%
14-40	T2	13	85.5	19.9	0%
14-43	T2	13	8.0	79.0	0%
14-98	T2	13	6.9	7.3	0%
15-13	T2	13	7.1	NDL	0%
15-26*	T2+T6	13+T6	58.5	4.5	NP
15-38	T2	13	1.0	0.7	0%
17-14	T2	13	5.4	NDL	0.03%
17-49**	T2+T6	13+T2	8.5	41.5	NP
17-64	T2	13	32.5	18.1	NP
11/26	T2	13	15.4	NDL	0%
W10-013	T2	13+T2	19.8	3.04	NP
W10-112	T2	13	6.9	4.8	0%
WV11-093	T2	13	12.8	1.5	NP

13= New Species; LPG= Larvae per gram; L/D= Ratio of number of live (coiled) and dead (comma shaped) larvae,

NDL= No dead larva found, NP= Not performed; * 3 larvae were T2+T13 (T2 on nuclear and T13 on mitochondrial designation, respectively); other

2 larvae were T2+T6 hybrid, and T6+T6.; ** 4 larvae were T2+T13; 1 larva was T6+T2 hybrid

Based on our findings, we hypothesize that T13 emerged prior to all encapsulated species (excluding *T. spiralis* + *T. nelsoni*) now distributed in the Holarctic (Eurasia and North America) and the Western Hemisphere, and may represent the outcome of an historical expansion event into North America from Eurasia through Beringia. Further studies on Alaskan and Siberian isolates of *Trichinella* may be helpful to resolve the complex history. Our phylogenetic analysis showed similar positioning of the previously described 12 species/genotypes of *Trichinella*; among the encapsulated clade, *T. spiralis* was the basal taxon, and non-encapsulated clade species infecting birds were in a distinct sub-clade from those infecting reptiles [183, 185]

To determine the host and geographical range of *Trichinella* (T13), 95 isolates purported to be *T. nativa* were re-tested. Specimens of T13 were found only in wolverines (out of 11 animal host species) from the Yukon and Northwest Territories (out of 7 provinces and territories of Canada). Prevalence of T13 was higher in wolverines from the Yukon than from the Northwest Territories, possibly due to sample bias. Further studies with more uniform sampling are required to determine if T13 is found only in wolverines from northwestern Canada. As well, the isolates of *T. nativa* were obtained by artificial digestion of tongue tissues. Though most species of *Trichinella* have predilection for tongue, diaphragm and leg muscles in wild animals [138-140, 155], we do not know the predilection sites for T13 in mammalian hosts. This factor could account for the lack of detection of T13 in mammalian hosts other than wolverines, as well as low prevalence (3%, 14/471) in wolverine. For millions of years, species of *Trichinella* have been undergoing adaptations to new geographical regions as well as hosts due to their opportunistic nature [144]. We hypothesize that T13 has higher infectivity in wolverines than other host animals, in part supported by the high larval intensities (>10 LPG) in half of the 14 wolverines positive for T13 (Table 4-4). Infectivity of the parasite plays a key role in establishment of muscle larvae in a host, and difference in infectivity among genotypes has been previously reported in pigs and wild boars, which are excellent hosts for *T. spiralis*, but are unsuitable hosts for *T. nativa* [126, 143, 188].

Trichinella (T13), *Trichinella* T6, and *T. nativa* occur in sympatry geographically (Yukon, Northwest Territories) and in the same host (wolverine) species. Sympatry of *Trichinella* T6 and *T. nativa* has been previously reported in wildlife from Alaska and wolverine from Nunavut, consistent with their designation as discrete species which maintain their genetic distinction in zones of contact and broader sympatry; however, hybrids have been reported, but without

significant introgression [67, 171]. Co-infections with different species could be acquired through single species infections acquired more than once during their lifetime, or concurrently from a single/separate source. The discovery of an undescribed species of *Trichinella* increases our understanding of species richness for a *Trichinella* fauna circulating in wolverine. These, and possibly other mustelids and carnivores, can act as reservoir hosts for five species of *Trichinella*; *Trichinella* T6, *T. nativa* (described in chapter 3), *T. spiralis* (Appendix A), *T. pseudospiralis* (Appendix B) and *Trichinella* T13.

Multiplex PCR commonly used in the identification of *Trichinella* isolates misidentified T13 as *T. nativa*, highlighting the need for sequencing, ideally of multiple loci or larger regions of the genome, to detect cryptic species. Failure of the current PCR methodology in species recognition of *Trichinella* has consequences, and suggests that prior identification (and reports) of *T. nativa*, and perhaps other species, may not completely reveal the extent of parasite diversity in some hosts and at varying localities. Larvae of species of *Trichinella* cannot be differentiated based on their morphology. Identification of species or genotype of *Trichinella* larvae can only be done by molecular methods [102-105]. Multiplex PCR is the most commonly used molecular method and is considered to be the standard approach in diagnostics. It is inexpensive and rapid, and targets internal transcribed spacers ITS1 and ITS2 and the expansion segment V region of the rRNA [102]. It produces a 127 bp size band diagnostic of *T. nativa*, which is also shared by T13. The PCR-RFLP developed in this study can differentiate between the isolates of *T. nativa* and T13. Further studies are required to design new primers for multiplex PCR or a new PCR methodology to differentiate species of *Trichinella* in North America and globally. We also recommend the use of newly developed PCR-RFLP to confirm the identity of T13 or *T. nativa*, or to use sequencing methodology to confirm the species of isolates of *Trichinella*.

Our phylogenetic analysis and compressorium examinations of the muscle tissues placed T13 in the encapsulated clade. Moreover, this species of *Trichinella* appears to have freeze resistance based on the fact that almost all of the positive animals had tightly coiled larvae, which morphologically indicates viability. Only one animal had viable larva based on the motility test. Further mouse inoculation tests are required to confirm the viability and pathogenicity of T13, relative to other species of *Trichinella*.

4.7 Conclusion

We report discovery of a cryptic species of *Trichinella*, designated as T13, that appears to be ancient and not closely related to existing members of the encapsulated species of the genus

from North America. Our results indicate that T13 is not geographically widespread in Canada, and appears limited to wolverine from Northwestern Canada, especially the Yukon, suggesting a possible link to Beringia and Eurasia. Further characterization of Alaskan and Russian isolates may be helpful in resolution of a complex history for biogeography involving expansion events linking Eurasia and North America through Beringia. In phylogenetic analyses, T13 was the putative sister of a subclade of encapsulated *Trichinella* containing *T. patagoniensis* and the remaining species of encapsulated group, excluding *T. spiralis* and *T. nelsoni*. Finally, our work revealed that the current multiplex PCR universally deployed to distinguish among known genotypes and species of *Trichinella* is missing unrecognized genetic diversity. We recommend use of newly developed PCR-RFLP or sequencing methodologies to confirm the identity of isolates attributed to *T. nativa* or any species of *Trichinella*.

4.8 Acknowledgements

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TRANSITION STATEMENT CHAPTER 5

In the first half of my thesis (chapters 2 to 4), I focused on *Trichinella*, an exclusively foodborne parasite. *Toxoplasma gondii* is another important food borne parasite in wildlife and people in the North; however, it has not been documented in the Yukon. It is therefore important to know the status of this parasite in wildlife of Yukon, and if wolverines would be a useful sentinel species for *T. gondii* infection as well as for *Trichinella* (Chapter 3). As clinical toxoplasmosis has rarely been reported in wildlife, and detection of *Toxoplasma* on gross necropsy examination is not possible, serological methods are usually used for screening live animals. However, commonly only dead animals are available in wildlife studies. Therefore, we used eluate from filter paper dipped in heart blood and fluid from thawed hearts in lieu of sera. Before screening wolverines for the presence of antibodies to *Toxoplasma*, I had to identify which specimen (filter elute vs heart fluid) and serological test (MAT, ELISA and IFAT) should be used. Likewise, I had to decide which tissue (brain or heart) should be tested for detecting *Toxoplasma* DNA in wolverines. In the current chapter, I compare the three most commonly used serological tests, and determine which tissue was most suitable for detection of *Toxoplasma* DNA using a sequence specific magnetic capture DNA extraction and real-time PCR method.

**CHAPTER 5 : COMPARISON OF TISSUES (HEART VS. BRAIN) AND
SEROLOGICAL TESTS (MAT, ELISA AND IFAT) FOR DETECTION OF
TOXOPLASMA GONDII IN NATURALLY INFECTED WOLVERINES (*GULO
GULO*) FROM THE YUKON**

Rajnish Sharma^{1*}, Sarah Parker², Batol Al-Adhami³, Nicholas Bachand¹ and Emily Jenkins¹

¹ Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

² Centre for Applied Epidemiology, Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

³ Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada, S7N 2R3.

Conceived and designed the experiments: RS, SP, EJ; Provided mentorship, logistics and supplies: SP, BA, NB, EJJ; Collection of samples: RS. Performed the experiments: RS; Analyzed the data: RS, SP, EJJ; Wrote the manuscript: RS, EJJ; Approved the final version of the manuscript: RS, SP, BA, NB, EJJ.

*Corresponding author: ras863@mail.usask.ca

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5.1 Abstract

Toxoplasma gondii is an important parasitic zoonosis worldwide. Many human and animal surveys use serological assays based on *T. gondii* antibody detection in serum, a matrix which is not routinely available from wildlife. Commonly used serological assays have rarely been validated for use with fluids other than serum, nor validated for their performance in wildlife species. New molecular assays, such as magnetic capture DNA extraction and real-time PCR (MC-qPCR), offer high sensitivity for detection of *T. gondii* DNA in tissues. The aims of this study were to (1) assess prevalence of *T. gondii* DNA based on MC-qPCR detection in brain and heart of naturally infected wolverines (*Gulo gulo*) from the Yukon, (2) compare two matrices (heart fluid and filter eluate) for antibody detection in the same species, and (3) evaluate the performance of three serological tests [modified agglutination test (MAT), enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT)] to detect naturally infected wolverines as determined by MC-qPCR. DNA of *T. gondii* was detected in heart and/or brain in 16 of 68 wolverines (24%, 95% CI: 15.0-34.8). Tissue prevalence and infection intensity was higher in heart [16 positives, 1221 tachyzoites equivalents per gram (TEG)] compared to brain (10 positives, 347 TEG). Heart fluid (HF) and filter eluates (FE) performed equally well in ELISA and IFAT in terms of sensitivity, but HF performed better with MAT. ELISA and IFAT had higher sensitivity (94%) and specificity (100%) compared to MAT (sensitivity 75% and specificity 92%). Overall, our findings indicate that the parasite burden in naturally infected wolverines was higher in heart compared to brain, heart fluid performed better than filter paper eluate for serological testing using MAT, and both IFAT and ELISA had higher sensitivity, specificity, and accuracy compared to MAT.

5.2 Introduction

Toxoplasma gondii infects almost all warm-blooded animals including humans, mammals and birds; one third of the global human population shows evidence of exposure to *T. gondii* [5, 6, 28]. Routes of transmission to people are (1) consumption of food or water contaminated with oocysts shed by felids, the only final hosts, (2) consuming raw/undercooked meat of infected animals containing bradyzoites in tissue cysts, and (3) via transmission of tachyzoites vertically from mother to fetus and (4) horizontally during blood transfusion and organ transplant [5, 27-31]. Therefore, animals play a key role not only in the life cycle of the parasite but also as a source of infection for people.

In northern Canada, seroprevalence of *T. gondii* varying from 6- 42 % has been reported in wild animals [42-44, 74]; however, to the best of our knowledge, no reports are available on the prevalence in the Yukon. Wild carnivores can act as sentinel animal species of *T. gondii* [73, 74] and can indicate whether *T. gondii* is present in the surrounding environment. Wolverines (*Gulo gulo*), fur-bearing mesocarnivores, have the potential to act as a sentinel animal host species, and a previous study showed that 42% of the wolverines (n=41) were seropositive to *T. gondii* in Nunavut, Canada based on the modified agglutination test (MAT) [42].

Methods that directly detect the parasite or its DNA in tissue indicate active infection in animals. These methods include bioassays, tachyzoite culture, immunohistochemistry and molecular techniques like PCR [110]. Cat and/mouse bioassays are the gold standard tests. Cat bioassay is a highly sensitive test as it uses 500 g of tissue; however, it is time consuming, expensive and has ethical issues [29, 110, 111]. Moreover, bioassays are not useful for previously frozen samples since testing relies on parasite viability [189], are not feasible for large-scale screening [190] and do not quantify infection intensity. A recently developed magnetic capture-qPCR (MC-qPCR), capable of analyzing up to 100 g of tissue [115] has successfully been used to detect *T. gondii* DNA in wild animals [74]. Thus, we selected MC-qPCR over conventional PCR methods due to higher sensitivity (it analyses 100 g vs 25-200 mg of tissue) and specificity (*T. gondii* DNA is selectively extracted using a specific probe bound to magnetic beads) [115]. Heart and brain are the most common predilection sites for *T. gondii* in animals [106, 111, 117, 118], but this has not yet been established for wolverines. Therefore, the first objective of the current study was to determine whether brain or heart is a more preferred site for *T. gondii* using MC-qPCR.

Indirect methods of detection are serological tests, which could indicate only exposure to *T. gondii* [106]. For serological tests, serum is the specimen of choice, but it is not always feasible to obtain serum samples from live or dead wild animals [107-109]. Several substitutes for serum in wild animal surveys primarily include blood, blood or body cavity fluid on filter paper, and meat juice [45, 74, 107, 108]; however, each specimen has its own merits and demerits. For example, collecting blood or cavity fluid on filter paper is preferred in remote areas where storage facilities are not available [108, 109]. Examining blood/cavity fluid or meat juice rather than filter paper eluate may be advantageous in terms of detecting low antibody levels. Meat juice and filter eluate have been used in several wildlife studies for detection of anti-*T. gondii* antibodies [45, 74, 107, 108]. Comparative studies on the performance of meat juice vs. filter eluate in wild animals are not available. Therefore, our second objective was to compare meat juice and filter eluate collected from wolverines using three serological tests, namely MAT, enzyme-linked immunosorbent assay (ELISA), and indirect fluorescent antibody test (IFAT).

Commercially available kits for the detection of anti-*T. gondii* antibodies are generally not validated for use in wildlife. Therefore, their performance in the target species should be evaluated before their use in sero-surveys. Previously, in-house MAT had been used in a *T. gondii* survey in wolverines in Nunavut; 1:25 cut off was used without any validation [42]. Therefore, the third objective of the current study was to compare the performance of MAT, ELISA and IFAT to detect anti-*Toxoplasma gondii* antibodies in naturally exposed/infected wolverines relative to MC-qPCR. Our findings will be helpful for wildlife disease researchers, and public health practitioners to select tissues, serological specimen and tests for future wildlife as well as public health surveys on *T. gondii*.

5.3 Methodology

5.3.1 Wildlife samples

Sixty-eight wolverine carcasses, submitted by Yukon fur trappers to Environment Yukon, were necropsied. For each wolverine, whole heart and brain were placed individually in double plastic bags to avoid cross-contamination between samples, and bags were labelled with wolverine ID number. Thoracic fluids were collected on five Nobuto filter paper strips (Advantec MFS, Inc, Dublin, CA, USA). After allowing to dry at room temperature overnight, filter paper strips were kept in individual envelopes, and stored at -20°C. The samples were shipped to the Department of Veterinary Microbiology at the University of Saskatchewan, and stored at -20°C until further analysis.

5.3.2 Collection of heart fluid (HF)

One ml of accumulated tissue fluid was collected from the bags containing thawed hearts, and centrifuged at 1000 g for 5 min at 4°C. The supernatant was collected in a 1.5 ml Eppendorf tube, and stored at -20°C.

5.3.3 Elution of Nobuto filter paper

The blood-soaked areas of two Nobuto filter paper strips were cut into 7-8 pieces, placed in an Eppendorf tube containing 800 µl of Dulbecco's Phosphate Buffered Saline and kept at 4°C for 16 hours [108]. The resulting filter eluate (FE) was collected in a new labelled Eppendorf tube and stored at -20°C.

5.3.4 Detection of *T. gondii* DNA in tissues using MC-qPCR

5.3.4.1 Extraction of *T. gondii* DNA

The whole heart and brain were thawed at 4°C for 24 hr. Then, connective tissue and fat was removed using a new scalpel blade and forceps for each organ. Magnetic capture-DNA extraction method was used to extract DNA from the whole heart and brain individually [115]. For each batch of DNA extraction, two spiked beef samples (2.5×10^5 tachyzoites/100g and 2.5×10^6 tachyzoites/100g) were used as positive controls and a beef sample without spiking was used as a negative control. Tachyzoites of *T. gondii* (VEG type III) were obtained from the Centre for Food-borne and Animal Parasitology (CFAP), Saskatoon. The DNA was stored at -20°C until further use.

5.3.4.2 Detection and quantification of *T. gondii* DNA

Real-time PCR was conducted in a thermocycler (Biorad, Hercules, California, USA) following the exact protocol as established by [74]. In brief, each reaction of 25 µL contained 0.5M iTaq DNA polymerase (Biorad, CA, USA), 10 µM of each primer (Tox 9F and Tox 11 R), 5 µM of a competitive internal amplification control (CIAC) probe, 2 fg of CIAC, 6.75 µL of PCR grade water and 8 µL template DNA. All reactions were run in duplicates; for each run, positive and negative extraction controls as well as no template controls were included. A dilution series of *T. gondii* plasmid DNA for obtaining a standard curve was also included in each run [74]. The reaction was considered as positive if (1) the Cp value was less than or equal to 35, (2) the positive control was positive, and (3) the negative and no template controls were negative. Samples with Cp values between 35 and 40 were considered positive if a 188 bp band was identified on gel electrophoresis. In case one of the duplicates or the CIAC did not amplify, the sample was repeated.

From the Cp values, quantification of parasites was done using the formula [74] : $\log_{10}(\text{tachyzoites}) = (43.3 - C_p) / 3.07$.

Quantification of parasites was expressed as number of tachyzoite-equivalents (TE) and termed as infection intensity.

5.3.5 Detection of *T. gondii* antibodies

5.3.5.1 Modified Agglutination Test (MAT)

The modified agglutination test was performed using a commercially available kit (New Life Diagnostic LLC, Carlsbad, CA, United States). Results were interpreted as per manufacturer's instructions.

5.3.5.2 Enzyme Linked Immuno Sorbent Assay (ELISA)

Using commercially available ID Screen® Toxoplasmosis Indirect Multi-species kits (IDvet, Grabels, France), ELISA was performed as per manufacturer's instructions. Optical density (O.D.) values at 450 nm were recorded using an ELISA automated plate reader (Spectramax, Molecular Devices). S/P % was calculated by formula $S/P\% = (OD_{\text{sample}} - OD_{\text{negative control}} / OD_{\text{positive control}} - OD_{\text{negative control}}) \times 100$. Samples presenting an S/P % less than or equal to 40% were considered negative. Samples with an S/P ratio greater than or equal to 50% were considered positive. If the S/P ratio was between 40% and 50%, test outcome was considered doubtful and repeated.

5.3.5.3 Indirect fluorescent antibody test (IFAT)

The IFAT was performed using commercially available antigen-coated Teflon-masked slides from VMRD (Pullman, WA, USA) and protein A/G FITC conjugate (BioVision, Milpitas, CA, USA). For IFAT, the optimal concentration of protein A/G FITC-conjugate was determined by using its different dilutions: 1: 300, 1:600 and 1:1200 with different dilutions of heart fluid and filter eluate. The staining procedure was performed as per manufacturer's instructions and the slides were observed using a fluorescent microscope at 40-100X. The wells with diffuse or intact peripheral staining of tachyzoites were considered positive. The wells with only apical/no staining of tachyzoites were considered negative. The broken peripheral staining of tachyzoites was considered as doubtful and was repeated.

5.3.5.4 Assessment of test specificity

Each MAT and ELISA included positive and negative controls provided by the manufacturers. Serum samples positive and negative for anti-*T. gondii* antibodies obtained from experimentally infected pigs were used in each IFAT test. To check for cross-reactivity, serum samples that were positive and negative for antibodies to *Neospora caninum* (bovine) and

Hamondia hamondii (feline) were examined in each serological test. Reference positive and negative sera (for *T. gondii*, *H. hamondii* and *N. caninum*) were provided by the CFAP, Saskatoon.

5.3.5.5 Pilot study: Evaluation of serological test performance with wolverine samples

As commercially available kits and slides used in this study were not evaluated for their use in wolverines and neither for use with filter eluates, we conducted a pilot study with three objectives 1) to evaluate if cut-off values (MAT) and sample dilutions (ELISA) as per manufacturer's recommendation work for this animal host species, 2) to establish cut-off values for IFAT as the manufacturer provided no cut-off values, and 3) to compare HF and FE to detect antibodies to *T. gondii*. We selected nine animals for this pilot study: three wolverines were negative in MC-qPCR for both tissues (group-1 N, negative). Six wolverines were MC-qPCR positive for both tissues, and all had Cp value less than 30 for heart tissue. Three of the latter 6 had a Cp value more than 30 for brain (group-2 LP, low positive), and the other three had a Cp value less than 30 for brain (group-3, HP, highly positive). The MAT was performed using HF and FE with four dilutions [1:25 (recommended cut-off), 1:50, 1:100 and 1:200]. ELISA was performed using HF and FE with two dilutions [1:2 (recommended dilution for HF) and 1:4] as well as with no dilution. IFAT was performed using HF and FE with five dilutions (1:2 to 1:32) and the results were graded 1+ to 3+ based on fluorescence intensity.

5.3.6 Statistical Analysis

5.3.6.1 Comparison of heart and brain

The proportion of positive results for heart and brain samples were compared with techniques for matched samples. McNemar Chi-square tests were performed to compare the proportion of wolverines positive for *T. gondii* DNA from hearts and brains. The kappa value (k) was calculated to determine the agreement between detection using the heart and brain. As the dependent variable (infection intensity) was not normally distributed, we used the Wilcoxon signed rank test (suitable for paired data) to compare levels of TE in heart and brain.

5.3.6.2 Comparison of serological methods

All doubtful results on serological tests were considered negative in this study. Using MC-qPCR as the determinant of infection status, sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated for each serological test. The kappa value (k) was calculated to determine the agreement between detection of anti-*T. gondii* antibodies using MAT, ELISA and IFAT. Kappa values of ≤ 0.40 , 0.40–0.60, 0.61–0.80 and ≥ 0.81 were considered

to represent slight to poor, moderate to good, substantial and excellent agreement, respectively [137]. All statistical tests were performed using commercial statistical software (IBM SPSS, ver. 24; Armonk, New York, USA).

5.3.7 Ethical approval

As the animals were harvested for purposes other than research, this was considered Category A and exempt from Animal Research ethics review at the University of Saskatchewan. We worked closely with Government of the Yukon partners for wildlife research and export permits.

5.4 Results

5.4.1 Comparison of heart and brain

Out of the 68 wolverines tested, 16 (24%) were positive for *T. gondii* DNA in at least one tissue. Fifty-two wolverines were negative for *T. gondii* in both types of tissue. Ten wolverines were positive for *T. gondii* DNA in both heart and brain, and six were positive in heart tissue only. The proportion of positive hearts (23.5%, 16/68) and brains (14.7%, 10/68) was significantly different ($P=0.031$, McNemar test).

There was substantial agreement between the detection of *T. gondii* from the hearts and brains ($k=0.72$). The infection intensity in the hearts ($n=16$) varied from 0.35 to 70624 tachyzoites equivalents per gram (TEG) (median= 1221, 25th–75th percentiles= 186 to 7157), and in the brains ($n=10$) from 0.91 to 10755 TEG (median= 347, 25th–75th percentiles = 37.6-721.0). Of the 10 animals positive in both tissues, eight wolverines had higher TEG in heart than brain, whereas two had higher TEG in their brain. The hearts had a significantly ($P=0.047$, Wilcoxon Signed Rank test, $n=16$) higher median parasite burden than the brains.

5.4.2 Specificity of serological assays

We observed cross-reactivity with serum samples positive for *H. Hammondii* antibodies using MAT; however, no cross-reactivity was observed with serum samples positive for *N. caninum* antibodies using MAT, and serum samples positive for *H. Hammondii* or *N. caninum* antibodies using ELISA or IFAT.

5.4.3 Results of the pilot study

All 3 MC-qPCR negative wolverines (group-1N) were negative at all dilutions in all three tests, except for ELISA, where undiluted heart fluid and filter eluate from one of the wolverines (ID-18) reacted positively. We observed no discordance in qualitative (sero-positivity) and

quantitative (titer) results in group-3 (HP) using the MAT, but in group- 2 (LP) the MAT titer for HF was higher than for FE, and one MC-qPCR positive wolverine (ID-33) showed a negative result with FE, indicating that HF may be a better specimen for the MAT and that a cut off value of 1:25, as recommended by the manufacturer, can be used for HF.

Using ELISA in groups 2 (LP) and 3 (HP), there was no difference when HF and FE were compared at dilutions of 1:2 and 1:4. However, S/P% was consistently higher for HF than for FE (at both dilutions). The observed S/P% of HF was also higher at a dilution of 1:2 than at 1:4. Therefore, HF and FE can be used at the recommended dilution of 1:2 for ELISA, and HF is a better matrix than FE for detecting anti-*T. gondii* antibodies. Using IFAT in groups 2 (LP) and 3 (HP), results were similar between HF and FE at dilutions of 1:2 and 1:4, but fluorescence was more intense for HF than FE (at both dilutions) for two positive wolverines (IDs-52 and 56). At dilutions of 1:8 and 1:16, HF and FE were still positive, but fluorescence decreased to 1+. At a dilution of 1:32, the FE of two wolverines (IDs-52 and 56) was negative (data not shown in table). The results of IFAT indicated that the cut-off values of 1:2 and 1:4 can be used for both HF and FE. Results are summarized in *Table 5-1*

5.4.4 Comparison of three serological methods relative to MC-qPCR

Because HF had a higher observed S/P% (in ELISA), titer (in MAT) and fluorescence (in IFAT) than FE, and a false negative result (ID-33) was obtained from FE (in MAT), we compared results using MAT (dilutions from 1:25 to 1:100), ELISA (dilution of 1:2) and IFAT (dilutions of 1:2 and 1:4) on HF. The results are presented in *Table 5-2*. All the test parameters

Table 5-1 Comparison of heart fluid and filter eluate using serological methods (MAT, ELISA and IFAT) relative to magnetic capture qPCR.

Group	ID	MAT titer Cut-off 1:25		ELISA S/P% Cut-off 50% S/P					IFAT Cut-off 1+			
		HF	FE	HF	FE	HF	FE	HF	HF	FE	HF	FE
				UD ^{\$}	UD	1:2	1:2	1:4	1:2	1:2	1:4	1:4
1 N	18	N	N	64.2	54.6	17.8	12.8	15.3	N	N	N	N
	38	N	N	27.5	33.3	8	4.7	7.8	N	N	N	N
	72	N	N	9.5	13.6	5.8	1.7	7.9	N	N	N	N
2 LP	12	1:100	1:50	262.8	238	236.8	229.3	225.9	3+	3+	3+	3+
	33	1:25	N	209.7	177.5	172.7	148.2	149.7	3+	3+	3+	3+
	56	1:200	1:100	277.3	238.8	262.8	228.4	244.7	3+	2+	3+	2+
3 HP	9	1:200	1:200	272	248.4	262.3	241.8	240.9	3+	3+	3+	3+
	34	1:50	1:50	223.9	242.9	204.7	200.2	188.4	3+	3+	3+	3+
	52	1:200	1:200	254	256.1	245.1	241.8	229.2	3+	2+	3+	2+

1 N= Negative on MC-qPCR, 2 LP = positive on MC-qPCR on heart but low intensity in brain, and 3 HP = highly positive on MC-qPCR on heart and brain; \$: This row contains dilutions of specimen (HF or FE) used ; FE -Filter eluate; HF - Heart fluid; UD-Undiluted; N- Negative; MAT- Modified agglutination test; ELISA- Enzyme linked immuno sorbent assay; IFAT- Indirect fluorescent antibody test

Table 5-2 Detection of *Toxoplasma gondii* antibodies in wolverine using different serological methods (MAT, ELISA and IFAT) compared to infection status as determined using the magnetic capture qPCR method.

	Number of tested samples		
	MC-qPCR		
Serological assay	Positive	Negative	Total
MAT			
Positive	12	4	16
Negative	4	48	52
Total	16	52	68
McNemar Test (p Value)	0.125	0.125	
IFAT			
Positive	15	0	15
Negative	1*	52	53
Total	16	52	68
McNemar Test (p Value)	1	-	
ELISA			
Positive	15	0	15
Negative	1*	52	53
Total	16	52	68
McNemar Test (p Value)	1	-	

MC-qPCR- Magnetic Capture-qPCR

MAT- Modified agglutination test

ELISA- Enzyme linked immuno sorbent assay

IFAT- Indirect fluorescent antibody test

*This wolverine had Cq value more than 35 on heart tissue, and was negative on brain tissue

(sensitivity, specificity, positive predictive value, negative predictive value and accuracy) were higher for ELISA and IFAT than for MAT (Table 5-3). Antibodies to *T. gondii* were detected in 16 (23.5%) of 68 wolverine using MAT, and 15 (22%) of 68 using ELISA or IFAT, respectively. In comparison to MAT (which incorrectly identified 4 tissue negative animals as positives), both ELISA and IFAT correctly identified more animals (15 vs. 12) that were positive in MC-qPCR (Table 5-2); thus, ELISA and IFAT had higher sensitivity (94%) and specificity (100%) than MAT (75% and 92%). ELISA and IFAT showed excellent agreement (Kappa=0.96) with MC-qPCR, whereas MAT showed only moderate agreement (Kappa=0.67). As we did not test HF with dilutions lower than 1:25 using MAT, false negatives could occur due to low detection level of antibodies at higher dilution. Moreover, the highest dilution used was 1:100, and high concentration of antibodies (prozone phenomenon) can also lead to false negatives in MAT. Therefore, to rule out these two possibilities, we retested the HF samples, which on MAT were doubtful and had discrepant results with MC-qPCR, and dilutions of HF used were from 1:12.5 to 1:800. We obtained similar results.

5.5 Discussion

In the present study, DNA of *Toxoplasma gondii* was detected in heart of wolverines more commonly than in brain. This finding is similar to that reported on the bioassay of experimentally infected dogs and foxes [191] [192]. Our study also found that heart had higher infection intensity (number of tachyzoite equivalents, TEG) than brain in naturally infected wolverines. This is an advantage of the MC-qPCR over bioassays, which could not quantitatively evaluate the parasite burden in these tissues. In addition to higher positivity and parasite burden in hearts than brains, collecting heart (vs brain) is easier, less time consuming, requires less expertise, can provide tissue fluid for serology, and poses less risk of rabies. Therefore, heart can act as a better sampling site than brain for surveys to detect *T. gondii* in naturally infected wolverines. Our study and others demonstrate the utility of MC-qPCR to measure infection intensity for *T. gondii* as it provides both qualitative and quantitative results [111, 118, 190, 193]. Animal host species, infective stage, inoculation dose, time after inoculation (acute vs chronic phase) and genotype can affect the infection intensity and predilection sites [117, 118, 190]. Further work should include MC-qPCR on other tissues (such as skeletal muscle) to determine the predilection sites for *T. gondii* in wolverine, as well as genetic characterization of *T. gondii* to determine if different genotypes have different tissue predilections.

Table 5-3 Comparison of MAT, ELISA and IFAT to detect *Toxoplasma gondii* antibodies in naturally infected wolverines

Assay	K	p ^{mt}	Sensitivity	Specificity	PPV	NPV	Accuracy
MAT	0.67	1	75 (51-90)	92 (82-97)	75 (51-90)	92 (82-97)	88 (79-94)
IFAT	0.96	1	94(72-99)	100 (93.1-100)	100 (93.1-100)	98 (90-100)	99 (92-100)
ELISA	0.96	1	94 (72-99)	100 (93.1-100)	100 (93.1-100)	98 (90-100)	99 (92-100)

*Parentheses have 95% CI

K- Kappa

p^{mt}- p value for McNemar test

PPV- Positive Predictive Value

NPV- Negative Predictive Value

MAT- Modified agglutination test

ELISA- Enzyme linked immuno sorbent assay

IFAT- Indirect fluorescent antibody test

Results of our pilot study showed that both HF and FE could be used to detect anti-*T. gondii* antibodies, but FE could give false negatives. Indeed, one MC-qPCR positive wolverine (ID-33) was sero-positive with HF, but not with FE. Likewise, discrepant results between FE and serum using MAT were reported in cats [194]. Better performance of HF than FE (consistently higher S/P%, fluorescence intensity, and MAT titer) in the current study could be due to higher levels of anti-*T. gondii* antibodies in HF than FE. This may in part reflect that MAT was performed on relatively more diluted HF and FE, as we made two fold dilutions starting at 1:25 for MAT, but started at 1:2 dilutions for IFAT and ELISA. The ELISA kit used in this study suggests 1:10 dilution for serum and 1:2 for HF, and previous studies showed that serum was considered to have a higher level of anti-*T. gondii* antibodies than meat juice; therefore, meat juice should be diluted less (1:2 vs. 1:10) than serum [195, 196]. Instruction manuals of commercial tests used herein indicate their intended use for serum, blood and meat juice, but have no information on their use for FE.

The manufacturers' recommended cut-off values and dilutions of HF [for MAT (cut-off: 1:25) and for ELISA (cut-off: S/P% greater than or equal to 50, dilution: 1:2)] seem appropriate for detecting anti-*T. gondii* antibodies in wolverines. Cut-off and dilutions of FE similar to HF can be used for ELISA and IFAT, but a choice for cut-off of FE in MAT requires further investigation. As our findings were based on small sample size (9 wolverines), further studies with large sample size are required.

Using MC-qPCR to determine infection status, ELISA and IFAT performed better than MAT in terms of sensitivity, specificity, PPV, NPV, and accuracy (*Table 5-3*). In comparison to MAT, ELISA and IFAT showed no false-positives (4 vs. 0) and fewer false-negatives (3 vs. 1), and therefore ELISA and IFAT had higher sensitivity and specificity. There were minimal chances that dilutions we used (1:25 to 1:100) were accountable for false negatives, but there is still a possibility that HF can be positive at lower dilution than the lowest dilution used (1: 12.5) in MAT; hence, further investigation is required. Previously, 1:10 dilution of meat juice has been suggested as a cut off using MAT to detect anti-*T. gondii* antibodies in experimentally infected pigs [195], similar to the 1:12.5 dilution used in the current study.

There could be several reasons for false positives reported in MAT in our study. First, the *H. hammondii* positive control serum showed cross-reactivity with *T. gondii* in MAT in this and previous studies [197, 198]. This cross-reactivity could be due to some common antigens shared by two parasites [199], and the positives we detected in MAT could have been infected with *H.*

hammondii. Second, MC-qPCR could be giving a false negative, if the amount of parasite was below the detection limit of the test, or the parasite was present in tissues other than heart or brain. Previously, bioassays, DNA detection methods and serological tests or their combination have been used to evaluate performances of serological tests [200]. Recently, serological tests have been compared in relation to MC-qPCR [201]. MC-qPCR is more sensitive than conventional PCR techniques, but may be less sensitive than a cat bioassay, which is the gold standard test for detection of *T. gondii* [28]. This comparison has not been done directly on the same samples. Since MC-qPCR has a low limit of detection of 4.45 tachyzoites/ g tissue [74], we used both heart and brain (most common predilection sites in other species), and ELISA and IFAT showed no positives on those four samples, it is more likely that MAT is giving a false positive.

In comparison to MAT, ELISA and IFAT are less laborious and time consuming and results are available on the same day. However, they require special instruments such as an ELISA reader and fluorescent microscope. Another disadvantage can be the prerequisite of species-specific conjugate, which are not available commercially mostly for wildlife. This is likely why the MAT (or DAT), which does not require species-specific conjugate, has been the most widely used test to detect anti-*T. gondii* antibodies in wildlife. However, this is addressed in the current study by using a protein A/G conjugate in both ELISA and IFAT, which binds to the IgG of various mammalian species, and has successfully been used for detection of anti-*T. gondii* antibodies in several species [62, 202, 203]. This is the first study to use protein A/G in IFAT to detect anti-*T. gondii* antibodies in wolverines. Our results add to the evidence that mammalian generalized protein A/G conjugate can be used in serological tests for multiple species for which commercial conjugates are not available. In summary, ELISA and IFAT were in excellent agreement, and either can be used for future surveys; in addition, ELISA results are automatically read, making them more objective than MAT or IFAT results.

5.6 Conclusion

Heart was a better sampling site than brain for detection of *T. gondii* DNA in wolverines due to the following findings: (1) a higher parasite burden detected, (2) more practical sampling site than brain, and (3) concurrent availability of heart fluid that can be used for comparative serological testing. However, further studies are required to identify other predilection or sampling sites. As serum from wild animals is usually unavailable, heart fluid can be a good candidate matrix to detect anti-*T. gondii* antibodies in wolverines using ELISA, IFAT and MAT, but cut-off values and false positives (low specificity) need to be further investigated for MAT. Based on our

findings, HF was preferred over FE to avoid false negatives. Further comparison between HF and FE should be studied with larger sample size. We suggest evaluating the performance of commercially available kits before their usage to detect *T. gondii* antibodies in wildlife species. As experimentally infected animals are generally lacking for wildlife species, sensitive tests to determine infection status of naturally infected animals should be used; for example, MC-qPCR, when bioassay is not feasible (frozen tissues, large scale studies) or desirable (animal research ethics).

5.7 Acknowledgements

We are grateful to the Yukon fur trappers that submitted wolverine carcasses for this study; without their involvement this project would have not been possible. We thank the many staff and students who assisted with obtaining and sampling the carcasses, particularly Jane Harms, Thomas Jung, Piia Kukka, Meghan Larivee and Philip Merchant (Government of Yukon). We also acknowledge Brent Wagner, Champika Fernando and Cherise Holding (Western College of Veterinary Medicine), Brad Scandrett and Yunxiu Dai (Canadian Food and Inspection Agency) for answering technical queries. We also thank Caroline Frey (Canadian Food and Inspection Agency) for reviewing and editing the manuscript. Funding for this study was provided by the Western College of Veterinary Medicine (University of Saskatchewan) Interprovincial Fund and Wildlife Health Research Funds, Government of Yukon, and a Discovery Grant and Northern Research Supplement (NRS) from the National Science and Engineering Research Council (NSERC).

TRANSITION STATEMENT CHAPTER 6

In the previous chapter, I compared three serological tests and two sera substitutes, and based on these results, decided to use ELISA on heart fluid to screen wolverines for the presence of *Toxoplasma* antibodies. Thus, in this chapter, I document the first report of risk factors associated with *Toxoplasma* exposure and infection in wolverines of Yukon. Also, I discuss the detection of *Toxoplasma* DNA in hearts of wolverines, which has public health significance, since handling/harvesting wildlife has been reported as a risk factor for human exposure to *T. gondii* in previous epidemiological studies. This information will be important for health authorities as well as trappers/harvesters to further implement and adopt preventative measures.

CHAPTER 6 : DETECTION OF ANTIBODIES TO AND DNA OF *TOXOPLASMA GONDII* IN FREE-RANGING WOLVERINE (*GULO GULO*) FROM THE YUKON, CANADA

Rajnish Sharma^{1*}, Jane Harms², Tom Jung², Piia Kukka², Sarah Parker³, Nick Bachand¹, Batol Al-Adhami⁴, and Emily Jenkins¹

¹ Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

² Department of Environment, Government of Yukon, P.O. Box 2703, Whitehorse, Yukon, Y1A 2C6, Canada

³ Centre for Applied Epidemiology, Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

⁴ Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada, S7N 2R3.

Conceived and designed the experiments: RS, JH, EJJ; Provided mentorship, logistics and supplies: JH, TJ, PK, SP, NB, BA, EJJ; Collection of samples: RS, JH, TJ, PK; Performed the experiments: RS; Analyzed the data: RS, SP; Wrote the manuscript: RS, EJJ; Approved the final version of the manuscript: RS, JH, TJ, PK, SP, NB, BA, EJJ.

*Corresponding author: ras863@mail.usask.ca

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6.1 Abstract

Toxoplasma gondii, an important parasitic zoonosis worldwide, has been documented in wildlife across northern North America, but has not been studied in wildlife in the Yukon. We used an Enzyme Linked Immunosorbent Assay (ELISA) to determine the prevalence of antibodies, and a sequence-specific magnetic capture DNA extraction and real-time PCR (MC-qPCR) to detect DNA of *T. gondii* in wolverines in the Yukon. Antibodies to *T. gondii* were detected in 27.5% (95% CI 23.0-32.5%) of 338 heart fluid samples. Antibodies were detected most commonly in adult wolverines (39%, 95% CI= 30.2-47.8), followed by yearlings (35%, 95% CI= 26.6-45.4) and juveniles less than 1 year of age (11%, 95% CI= 6.8-18.0). DNA of *T. gondii* was detected in heart tissues of 24.6% (95% CI= 20.3-29.4) of 338 wolverines. Mean infection intensity was 6454 tachyzoite equivalents per gram of tissue (TEG) (95% CI=3462-94450). The odds of presence of anti-*T. gondii* antibodies in heart fluid and DNA in heart tissue were 4-5 times higher in yearlings and adults, respectively, than in juvenile wolverine. Eighty-one wolverines were positive on both ELISA and MC-qPCR. Strong correlation between the results of ELISA and MC-qPCR, including quantitative measures of antibodies and DNA, supports the use of ELISA in wild carnivores when tissue samples are not available or feasible; for example, in endangered species or animals captured live for collaring studies. Overall, prevalence of *T. gondii* in wolverines, a sentinel species, in the Yukon was lower than that reported in this species elsewhere in northern Canada, which suggests lower amounts of the parasite circulating in wild and domestic animals, and corresponds with lower seroprevalence in people in the western North American Arctic. Future studies are needed to determine the status of viable parasite in tissues, and genotypes of *T. gondii* circulating in wolverines of the Yukon.

6.2 Introduction

Toxoplasma gondii ranks 4th among the 24 significant foodborne parasites listed by FAO/WHO [3]. It is considered one of the most successful parasites, as one third of the world's human population has been exposed to *T. gondii*, and almost all warm-blooded animals can become infected with *T. gondii* [6, 28]. *Toxoplasma gondii* usually does not cause any clinical disease in immunocompetent people, but can lead to serious clinical manifestations in the fetuses of pregnant woman, or people such as AIDS patients. People can acquire *T. gondii* infection by three routes: (1) consumption of food or water contaminated with oocysts excreted by felids, the only known definitive hosts; (2) ingestion of raw or undercooked meat (containing tissue cysts) of other intermediate hosts, and (3) via transplacental transmission from infected mother to fetus [28]. Domestic or wild animals are therefore essential for the life cycle and transmission of *T. gondii*.

Wild animals play a key role in transmission and persistence of *T. gondii* in Canada, especially in northern regions, where they have been linked to outbreaks of human toxoplasmosis. For example, in an outbreak of congenital toxoplasmosis in Quebec, Canada, frequent consumption of caribou meat was a significant risk factor for acquiring infection [92]. Contamination of municipal drinking water with feces of cougars (*Felis concolor vancouverensis*) as well as domestic cats was the presumed reason for another outbreak in British Columbia, Canada [94]. *Toxoplasma gondii* can also affect health of animals; e.g. abortion and congenital toxoplasmosis in a captive reindeer [204], and fatal disseminated toxoplasmosis in captive meerkats (*Suricata suricatta*) [205], and wild Arctic foxes (*Alopex lagopus*) [206] have been documented. Due to traditional, cultural and subsistence values, people in northern Canada have frequent contact with wildlife, which could increase the risk of *T. gondii* infection to humans, and to control such infections in people as well as animals, information on epidemiology of *T. gondii* in wild animals is therefore essential.

Several epidemiological studies have been conducted in wild animals in northern Canada, and prevalence of antibodies to *T. gondii* varied from 1-42% and 1-46% in northwestern (Nunavut, Northwest Territories and the Yukon) and northeastern Canada (Quebec and Newfoundland and Labrador), respectively [15]. To best of our knowledge, a single study has been conducted on caribou of the Yukon along with those from Alaska, but no information on number of sampled wildlife from the Yukon was stated [40]. Such epidemiological information on *T. gondii* in wildlife from the Yukon would not only be helpful for disease control and wildlife management policies, but also to generate baseline data for future studies.

Prevalence of *T. gondii* infection in terrestrial carnivores is usually higher than herbivores in northern Canada [15], and wild terrestrial carnivores can act as sentinel animal species of *T. gondii* [73, 74]. It is both cost-effective and time efficient to first study sentinel animal hosts rather than targeting multiple animal host species, especially herbivores. Wolverine (*Gulo gulo*) exhibit characteristics of an ideal sentinel host species including the following: (1) adequate availability (population stability): wolverine carcasses are available from ongoing, legal harvesting of wolverines in the Yukon; (2) measurable response (e.g. parasites in tissues, antibodies in blood): anti-*T. gondii* antibodies have been detected in wolverines [42]; (3) Exposure by routes of transmission similar to target hosts (people or game animals): wolverines are omnivores and can therefore contract infection by consumption of oocysts in contaminated water or vegetation and or tissue cysts in infected carrion or prey; (4) high levels of exposure: being in an apex position in the food chain, wolverines bio-accumulate foodborne parasites such as *Trichinella* spp. and *T. gondii* and (5) earlier response than people/co-habiting animals (due to short life span) [75, 76]. Therefore, in order to establish presence of *T. gondii* in the environment and wildlife in the Yukon, the first objective was to determine seroprevalence of anti-*T. gondii* antibodies and associated risk factors in naturally infected wolverine in the Yukon.

Serological methods [e.g modified agglutination tests (MAT), indirect immune fluorescent test (IFAT), enzyme linked immunosorbent assay (ELISA) etc.] detecting antibodies only demonstrate if an animal has been exposed at some point in its life, but cannot demonstrate current *T. gondii* infection [106]. Most wildlife studies have relied on serological assays [42] rather than detection of *T. gondii* in tissues. The latter is more meaningful in assessing risks to wildlife and public health, indicating current levels of the parasite circulating in the environment, and quantifying the infection intensity rather than strength of the immune response as measured by antibody titres. Therefore, the second objective of the current study was to evaluate if *T. gondii* infection and intensity (indicated by number of tachyzoite equivalents of *T. gondii* DNA in tissues) were associated with serological assay results and the same risk factors as investigated for the serological results.

6.3 Material and methods

6.3.1 Study area and samples

The Yukon is a sparsely populated territory located in northwestern Canada, bordered by the Northwest Territories and British Columbia in Canada, and Alaska in the USA [148]. The mean annual temperature and precipitation range from -2°C to -10°C and 250 to 600 mm, respectively

[149]. The Yukon has a wide range of habitats, from arctic and alpine tundra to boreal forest. Wolverines range throughout the Yukon. Based on an estimated wolverine population size between 3500–4000 animals in the Yukon [49], and an expected 42% seroprevalence of *T. gondii* in wolverines, based on a previous study in Nunavut [42], the calculated sample size required for the current study was 341 with a confidence interval of 95% using OpenEpi ver. 3.01 [154].

Wolverines are legally harvested for fur in Yukon, and on average 132 (\pm 31 SD) wolverines are harvested annually [148, 150, 151]. Wolverine harvesters are encouraged to submit their skinned wolverine carcasses to the government of the Yukon. Submitted carcasses are frozen at -20°C for 6–10 months prior to necropsy. We obtained 338 wolverine carcasses from animals harvested during 4 winters (2013–14 to 2016–17). During necropsy, whole heart from each wolverine was collected in plastic bags and stored at -20°C until further analyses. Origin of wolverine carcasses (northwest Yukon and southeast Yukon; *Figure 6-1*), and sex were recorded. A body condition index (BCI) [151], and age for each wolverine was determined by cementum analysis of premolar tooth at a commercial laboratory (Matson's Laboratory LLC, Milltown, Montana, USA); wolverine were classified as juveniles (<1 year), yearlings (1–2 years), or adults (≥ 2 year). As animals were harvested for purposes other than research, this was considered Category A and exempt from Animal Research ethics review at the University of Saskatchewan. Relevant wildlife research and export permits were obtained from the government of Yukon.

6.3.2 Detection of anti- *T. gondii* antibodies

6.3.2.1 Collection of heart fluid (HF)

The whole heart was thawed in a plastic bag at 4°C for 24 hr and 1 ml of accumulated tissue fluid was collected from the bag into a 1.5 ml Eppendorf tube. Heart fluid was centrifuged at 1000 g for 5 min at 4°C , and the supernatant collected and stored in a new 1.5 ml Eppendorf at -20°C until further testing.

6.3.2.2 Enzyme Linked Immunosorbent Assay (ELISA)

We performed ELISA using commercially available ID Screen[®] Toxoplasmosis Indirect Multi-species kits (IDvet, Grabels, France). Previously, we evaluated the performance of this kit for detection of anti-*T. gondii* antibodies in heart fluid obtained from wolverines; ELISA had 94% sensitivity and 100% specificity (Sharma et al, accepted to Food and Waterborne Parasitology). Heart fluids diluted to 1:10 were tested, and optical density (O.D.) was recorded at 450 nm using an ELISA automated plate reader (Spectramax, Molecular Devices). S/P % was determined by the formula: $\text{S/P\%} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}) / (\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}}) \times 100$. Samples with an

S/P% ratio less than or equal to 40%, greater than or equal to 50%, and between 40% and 50% were considered negative, positive, and doubtful, respectively. All samples were tested in duplicates, and in each ELISA test, positive and negative controls provided by the manufacturers as well as wolverines of known tissue status were included.

6.3.3 Detection of *T. gondii* DNA in heart tissue using MC-qPCR

From sero-positive wolverines, DNA was extracted from the whole heart (up to 100 g). From sero-negative wolverines, 20 g of heart tissue from each of five individual animals were pooled to make 100 g and processed. If a pool of heart tissue from sero-negative wolverines was positive, DNA was extracted and tested individually from the remaining heart tissue of respective wolverines.

6.3.3.1 Extraction of *T. gondii* DNA

Fat was removed from the heart using a new scalpel blade and forceps, and magnetic capture-DNA extraction method was performed to extract DNA [115]. Two spiked beef samples (2.5×10^5 tachyzoites/100g and 2.5×10^6 tachyzoites/100g) and a beef sample without spiking were used as positive and negative controls, respectively. Tachyzoites of *T. gondii* (VEG type III) were provided by CFAP, Saskatoon. DNA was kept at -20°C until further analysis.

6.3.3.2 Detection and quantification of *T. gondii* DNA

Real-time PCR was performed as per [74]. Briefly, each reaction (25 μL) contained 10 μM of each primer (Tox 9F and Tox 11 R), 5 μM of a competitive internal amplification control (CIAC) probe, 2 fg of CIAC, 0.5M iTaq DNA polymerase (Biorad, CA, USA), 6.75 μL of PCR grade water and 8 μL template DNA. Positive and negative extraction controls as well as no template controls were incorporated in each PCR run. The reaction was considered as positive if (1) the negative and no template controls were negative, (2) the positive control was positive, and (3) the C_p value was less than or equal to 35. Samples with C_p values between 35 and 40 were considered positive if a 188 bp band was identified on gel electrophoresis. Parasites were quantified using the following formula: $\log_{10}(\text{tachyzoites}) = (43.3 - C_p) / 3.07$, and expressed as number of tachyzoite-equivalents (TE) [74]. The intensity of infection was calculated by dividing tachyzoite-equivalents (TE) by weight of the heart processed and expressed as tachyzoite-equivalents per gram (TEG).

6.3.4 Statistical analysis

6.3.4.1 Seroprevalence, tissue prevalence, infection intensity and risk factors

Prevalence with 95% confidence intervals (CI) was calculated from the proportion of positive results using EpiTools epidemiological calculators [207]. Binary logistic regression was performed for outcome variables: (1) Exposure to *T. gondii* (presence or absence of anti-*T. gondii* antibodies using ELISA) (2) *T. gondii* infection (presence or absence of *T. gondii* DNA using MC-qPCR). Linear regression was performed to determine association between the infection intensity (outcome variable: TEG) and predictors. Potential predictors for inclusion in a final multivariable model using univariable regression analysis were: Age (juvenile, yearling and adult), sex (female and male), harvest location (southeast Yukon and northwest Yukon), harvest season (4 annual seasons), body condition index (continuous variable), *Trichinella* spp. infection (positive and negative), and *Trichinella* spp. identified [negative, *T. nativa*, *Trichinella* T6, mixed infection (both *T. nativa* and *Trichinella* T6)]. A relaxed level of significance ($p \leq 0.20$) was used to identify potential variables for consideration in multivariable analysis. Stepwise forward multivariable regression analysis was performed to build the final models. Goodness of fit of the final model was evaluated by the Hosmer-Lemeshow test. Variables associated at a significance level of $p < 0.05$ were retained in the model. To estimate the degree of the association between each predictor and presence of anti-*T. gondii* antibodies, odds ratios and respective 95% confidence intervals were calculated.

6.3.4.2 Comparison and relation between MC-qPCR and ELISA results

To determine if correlation exists (1) between probability of MC-qPCR positive (out come variable, Yes/No) and ELISA positive (Yes/No), (2) between probability of MC-qPCR positive (out come variable, Yes/No) and antibody concentration (independent variable), binary logistic regression was performed. Linear regression analysis was performed to evaluate the correlation between antibody concentration (S/P%) and Cp value obtained from MC-qPCR. All statistical analyses were performed using IBM SPSS (ver. 24; Armonk, New York, USA).

6.4 Results

6.4.1 Seroprevalence and associated risk factors

Anti-*T. gondii* antibodies were detected in 27.5% of wolverine tested (95% CI 23.0-32.5%). Anti- *T. gondii* antibodies were detected most commonly in adult wolverines (39%, 95% CI= 30.2-47.8), followed by yearlings (35%, 95% CI= 26.6-45.4) and juveniles (11%, 95% CI= 6.8-18.0). More proportion of females (33%, 95% CI = 25.0-42.1) than males (25%, 95% CI= 19.6-30.8)

were seropositive for *T. gondii*. Of 93 sero-positive wolverines, 85% were co-infected with *Trichinella* spp. (Table 6-1).

Our univariable logistic regressions revealed that age ($p < 0.001$), sex ($p = 0.111$), *Trichinella* spp. infection ($p = 0.046$) and *Trichinella* spp identified ($p = 0.124$) were significantly associated with *T. gondii* sero-positivity. Stepwise multivariable regression suggested that age was significantly associated with *T. gondii* sero-positivity. The odds of presence of anti-*T. gondii* antibodies were 4 times (odds ratio = 4.35, 95% CI = 2.17-8.72; $p < 0.001$) and 5 times (odds ratio = 4.98, 95% CI = 2.55-9.76; $p < 0.001$) higher in yearlings and adults, respectively, than in juvenile (<1 yr), wolverine.

6.4.2 Tissueprevalence and associated risk factors

Of 338 wolverines, *Toxoplasma gondii* DNA was detected in heart tissues of 83 wolverines (24.6% 95% CI= 20.3-29.4). Univariable regressions revealed that age ($p < 0.001$) and *Trichinella* spp. infection ($p = 0.161$) were significantly associated with presence of DNA of *T. gondii* in tissue, but only age remained significantly associated with *T. gondii* tissue-positivity on multivariable regression. The odds of presence of *T. gondii* DNA were 4 times (odds ratio = 4.370, 95% CI = 2.27-9.78; $p < 0.001$) higher in yearlings and adults than in juvenile (<1 yr) wolverine.

6.4.3 Intensity of infection and risk factors

Mean infection intensity was 6454 TEG (95% CI = 3462-94450). None of the predictors was associated with infection intensity on univariable regression analysis, and therefore multivariable model was not built. Mean (95% CI) and median TEG among different classes of predictor variables are shown in Table 6-1.

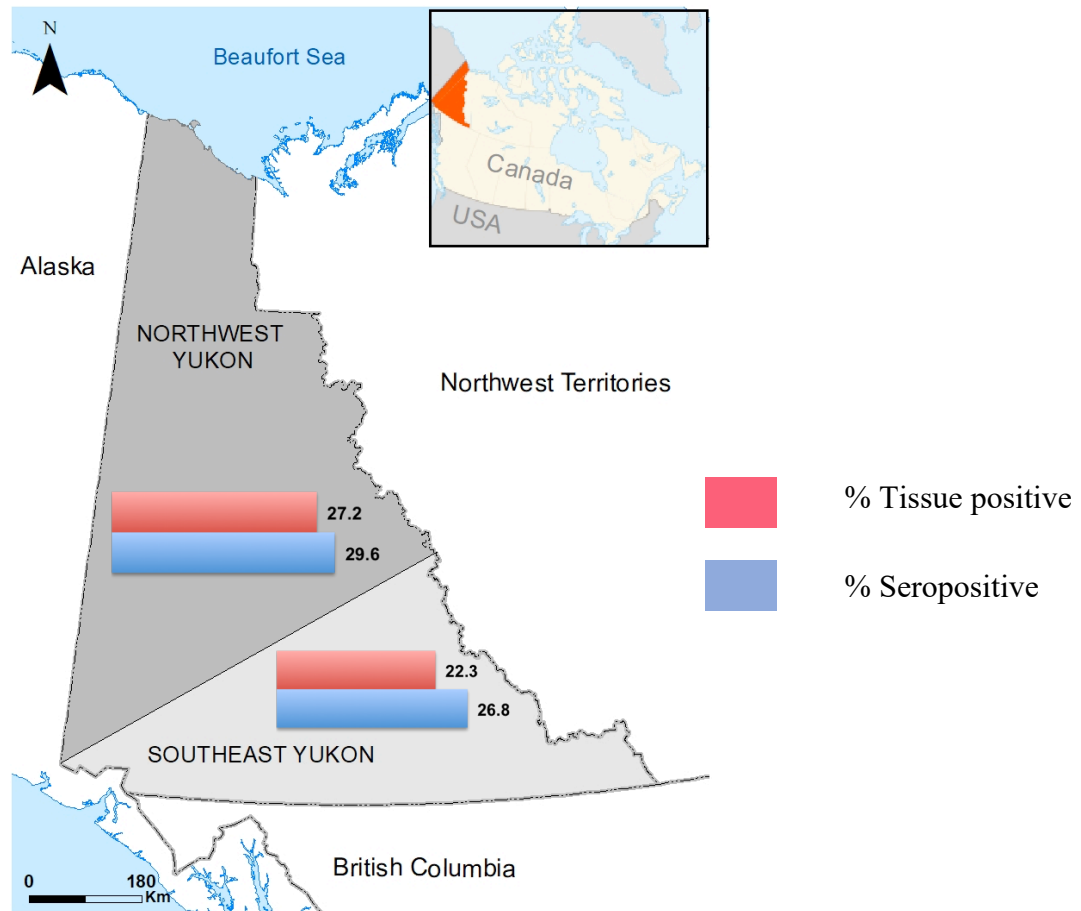


Figure 6-1 Prevalence of *T. gondii* in wolverines from two harvest locations of the Yukon

Table 6-1 Seroprevalence, tissue prevalence, and infection intensity of *T. gondii* in wolverine (*Gulo gulo*) from the Yukon

		TT	SP; (%; 95 % CI)	TP; (%; 95 % CI)	Mean TEG (95% CI); median
Age	Juvenile	125	14; 11.2 (6.8-18.0)	12; 9.6 (5.6-16.0)	4854 (607-9101); 1770
	Yearling	96	34; 35.4 (26.6-45.4)	32; 33.3 (24.7-43.2)	7925 (1714-14136); 867
	Adult	114	44; 38.6 (30.2-47.8)	38; 33.3 (25.4-42.4)	5669 (1645-9713); 1140
Sex	Female	112	37; 33.0 (25.0-42.1)	30; 26.8 (19.6-35.7)	8199 (3185-13212); 5738
	Male	226	56; 24.8 (19.6-30.8)	53; 23.5 (18.4-29.4)	5466 (1640-9292); 667
Harvest location	Northwest	169	50; 29.6 (23.2-36.9)	46; 27.2 (21.1-34.4)	7500 (3106-11892); 1607
	Southeast	157	42; 26.8 (20.4-34.2)	35; 22.3 (16.5-29.4)	5446 (1128-9764); 1054
Harvest year	2013-14	86	27; 31.4 (22.6-41.8)	23; 26.7 (18.5-37.0)	1183 (2655-19510); 4323
	2014-15	91	23; 25.3 (17.5-35.1)	19; 20.9 (13.8-30.3)	3637 (593-6681); 780
	2015-16	77	23; 29.9 (20.8-40.9)	21; 27.3 (18.6-38.1)	3189 (522-5856); 667
	2016-17	84	20; 23.8 (16.0-33.9)	20; 23.8 (16.0-33.9)	7234 (-166-14633); 1221
<i>Trichinella</i> infection	Negative	76	14; 18.4 (11.3-28.6)	14; 18.4 (11.3-28.6)	4534 (244-8825); 725
	Positive	262	79; 30.2 (24.9-36.0)	69; 26.3 (21.4-32.0)	6843 (3322-10364); 1226
<i>Trichinella</i> spp. identified	Negative	76	14; 18.4 (11.3-28.6)	14; 18.4 (11.3-28.6)	4534 (244-8825); 725
	<i>T. nativa</i>	22	9; 40.9 (23.3-61.3)	8; 36.4 (19.7-57.1)	2259 (-120-4639); 1094
	<i>Trichinella</i> T6	199	55; 27.6 (21.9-34.2)	47; 23.6 (18.3-30.0)	8401 (3368-13434); 1578
	Mixed infection	32	11; 34.4 (20.4-52.7)	10; 31.3 (18-48.6)	5699 (-57-11454); 1732
Total		338	93; 27.5 (23.0-32.5)	83; 24.6 (20.3-29.4)	6454 (3462-94450); 1093

TT= Total number of animals tested

SP= Number of seropositive animals

TP= Number of tissue positives

TEG= Tachyzoites equivalent per gram

%= Percentage proportion of positive animals

6.4.4 Relation between qualitative MC-qPCR and ELISA results

Eighty-one wolverines were positive on both ELISA and MC-qPCR, 12 were positive on serology but negative on MC-qPCR, and two were positive on MC-qPCR and negative on ELISA (Table 6-2). Binary logistic regression showed that the odds of being tissue positive was 820 (95% CI= 179.7-3742, $p < 0.001$) times higher in seropositives than seronegative wolverines. Probability of tissue positive (P) can be predicted from the SP% with a logistic regression equation [$\log(P/1-P) = -4.85 + 0.43 * S/P\%$], and model was fit (Hosmer and Lemeshow test $p = 0.670$; Chi square=5.80, d.f=8). ROC curve analysis showed that MC-qPCR outcome (positive or negative) could be predicted accurately using the aforementioned model (AUC=0.990, 95% CI= 0.983-0.998).

6.4.5 Relation between quantitative MC-qPCR and ELISA results

Linear regression performed on data from 83 tissue positive wolverines indicated significant correlation between antibody concentration (SP%) and infection intensity (Cp) [$Cp = 37.74 - 0.05 * SP\%$], and model indicated that the SP% could explain a moderate proportion of variation in the Cp value (Adjusted R square= 0.435)

6.5 Discussion

Our study is the first report of *T. gondii* in non-migratory wildlife in the Yukon, which suggests that the parasite is circulating in terrestrial ecosystems in this region. We detected lower prevalence of anti-*T. gondii* antibodies in the Yukon wolverines (27% of 338) in comparison to wolverines in Nunavut (42% of 41) [42], but higher than that in wolverines in BC (0% of 20) [68]. Prevalence in our study was also lower than in other terrestrial carnivores from the northern North America; for example, arctic foxes (*Vulpes lagopus*) from Alaska (59%) and Nunavut (39%); red foxes (*Vulpes vulpes*) from Quebec (41%); black bear (*Ursus americanus*) from Alaska (43%); and grizzly bear (*Ursus arctos*) from Alaska (67%) [74, 208-210]. On the contrary, seroprevalence was higher than that reported in wolves (*Canis lupus*) from Alaska (9-18%) [40, 210]. Differences in seroprevalence among wolverine populations and between wolverines and other wild hosts in northern North America could be due to several factors, including variation in the geographical origin, dietary habits, felid densities, *T. gondii* prevalence in wild/domestic felids, distribution of

Table 6-2 Comparison of detection of *T. gondii* antibodies and DNA in wolverine (*Gulo gulo*) from the Yukon

	MC qPCR positive	MC qPCR negative	Total
ELISA positive	81	12	93
ELISA negative	2	243	245
Total	83	255	338

prey species, and climatic variation affecting oocyst survival. Interestingly, our low prevalence in wolverine in the Yukon corresponds with observations of low human seroprevalence in Alaska and in the neighboring Inuvialuit Settlement region, much lower than in the central and eastern Canadian Arctic [93, 211]

As expected, age was significantly associated with *T. gondii* exposure, which could be due to more cumulative exposure in older wolverines through consumption of tissue cysts in infected carrion/meat and sporulated oocysts in contaminated food or water. Another reason could be persistence of the antibodies (possibly lifelong) in the exposed wolverines; however, we saw the same association with presence of DNA in tissues. It is not known how long humoral response to *T. gondii* in wolverines lasts, nor for how long tissue cysts can survive. Our age related results are consistent with observations in wolverines as well as other wildlife hosts, e.g. lynx (*Lynx canadensis*) from Quebec, Canada; Eurasian otter (*Lutra lutra*) from England and Wales [212]; wolves (*Canis lupus*) from Alaska [210] and maned wolves (*Chrysocyon brachyurus*) from Brazil [213]. In contrast, no significant association between age and *T. gondii* exposure was observed in wolverines from Nunavut [42], bobcats (*Lynx rufus*) from Quebec [214], and feral cats from Prince Edward Island, Canada [215].

Although sex was not a significant predictor for serostatus, there was a higher proportion of seropositive females than male wolverines in the current study. This is counter to expectation, as female wolverines, typically have smaller body size and home ranges than males (e.g. Dawson et al. 2010; Persson et al. 2010), and would presumably have decreased chance of exposure. We observed higher proportion of wolverines positive for *T. gondii* in northwestern vs southeastern Yukon, which could be attributed to higher environmental contamination with oocysts of *T. gondii*, and warrants further studies to determine if there are links between prevalence of *T. gondii* in wildlife and prevalence in lynx. In addition, there could be potential sources of infection that may originate outside our study area e.g. oocyst/tissue cyst transportation via migratory animals. Prevalence decreased slightly from seasons 2013-14 to 2016-17, and no significant difference in prevalence among different harvest seasons was observed.

We detected, for the first time, DNA of *T. gondii* in a wild mammal host in the Yukon. We utilized a direct detection method in addition to serology, which is a powerful approach that provides more information than most surveillance studies on *T. gondii* in wildlife, which use only serological methods indicating lifetime exposure. Recently, DNA of *T. gondii* has been detected

using MC-qPCR in red foxes (*Vulpes vulpus*) from Quebec, Canada [74]. MC-qPCR is less laborious, inexpensive, and quantitative as compared to animal bioassays. While bioassays are considered the gold standard method for detection of *T. gondii*, cat and mouse bioassays are not feasible on frozen meat samples, as *T. gondii* is presumed killed at freezing temperatures of -20C for greater than 3 days [189]. Strong correlation between the results of ELISA and MC-qPCR supports the use of ELISA in wild animals where tissue samples are not available or feasible; for example in endangered species, or live-captured wildlife. Presence of anti-*T. gondii* antibodies and absence of DNA in heart tissues of 12 wolverines could be due to either (1) possibility of tissue cysts at site(s) other than heart or (2) infection intensity in heart tissue lower than the detection limit of the MC-qPCR. Two wolverines were positive on MC-qPCR and negative on ELISA, which could be ascribed to acute infection, where tissue cysts of *T. gondii* could be present in tissues but antibodies were not yet produced, or chronic infections, where antibody response has faded but tissue cysts persist in a non-immunogenic state. New quantitative molecular techniques offer exciting opportunities for further study on relationship between tissue infections and dynamics of anti-*T. gondii* antibodies.

Wolverines are not consumed for food by people, but they are harvested for their fur. Skinning of fur animals has been reported as a risk factor for *T. gondii* exposure in people [92]. We recommend that fur trappers use caution when handling wildlife carcasses, and avoid leaving carcasses in the field after harvesting to prevent scavenging and perpetuation of the life cycle of this potential foodborne zoonosis. Further studies are required to determine the tissue distribution and viability of *T. gondii* present in wolverines and wildlife species consumed by people.

The current study, for the first time, determined the infection intensity, as measured by tachyzoite-equivalents per gram (TEG), of *T. gondii* in wildlife tissues; previous studies have used MC-qPCR to determine infection intensity in domestic livestock. This is still relatively unusual for microparasites such as protozoans. None of the demographic risk factors examined in this study (age, sex, location) were associated with infection intensity in the wolverines, which could be due to the small number of tissue positive wolverines, or low levels of variation in infection intensity of *T. gondii* in heart tissue of wolverines. However, we previously demonstrated that heart had higher TEG than brain from naturally infected wolverines (Sharma et al, accepted, Food and Waterborne Parasitology). Further studies are required to determine predilection sites in other organs and muscles, and their relation to overall infection intensity of *T. gondii*.

Wolverines can become infected with *T. gondii* via consumption of water or food contaminated with sporulated oocysts shed in the feces of wild cats. The ranges of Canadian lynx and wolverines overlap throughout the Yukon, whereas cougars (*Puma concolor*) are rare and restricted to the southern Yukon. Prevalence of *T. gondii* in felids has not been studied in the Yukon but has been reported in adjacent regions of Alaska and British Columbia; sero-prevalence is 15% in lynx in Alaska [214, 216], and 92% in cougars in British Columbia [94]. Wolverines could also be infected through consumption of tissue cysts in infected prey or carrion. Wolverines are opportunistic feeders that hunt or scavenge locally available prey or carrion, such as snowshoe hare, ungulates (e.g. moose and caribou), various rodents (e.g. sciurids and voles), birds and other carnivores (Robitaille et al. unpublished data). Anti-*T. gondii* antibodies have been reported in 1-23% of moose from Alaska and 1-29% of caribou (*Rangifer tarandus*) from Nunavut, Northwest Territories and Alaska [38, 41, 217]. Low prevalence of *T. gondii* antibodies (0.2%) in the Porcupine caribou herd from the Yukon and Alaska [40] suggests that there may not be significant infection pressure from oocysts shed by wild felids in this region of the Yukon. No data on *T. gondii* in snowshoe hare (common prey species for wolverine and lynx) in northern North America is available. It is possible that snowshoe hare are infected through consumption of contaminated vegetation or carrion and may subsequently transmit parasites to their predators, such as lynx and wolverine. Vertical transmission of *T. gondii* has been documented in a mustelid host [218], and is another potential route of transmission in wolverines. Further studies are required to determine the status of *T. gondii* in other wildlife of the Yukon to determine potential sources and transmission routes in wolverines.

Although beyond the scope of the current study, *T. gondii* has the potential to influence health and reproductive success of mustelids. An outbreak of toxoplasmosis has been reported in farmed mink (*Mustela vison*) in USA, where around 2000 female minks lost their entire litter either due to neonatal mortality or abortion [219]. Limb lameness, ataxia and bilateral blindness due to *T. gondii* infection in free ranging mink was also reported [84]. Clinical disease due to *T. gondii* has been reported in ferrets (*Mustela nigripes*) and southern sea otters (*Enhydra lutris nereis*) [83, 220]. We do not know if *T. gondii* causes clinical manifestations and reproductive loss in wolverines in the Yukon. Finally, it will be important to determine the genetic diversity of *T. gondii* in wolverine to determine if they are primarily infected with clonal types of *T. gondii* common in domestic animals and people in temperate regions, or if there are distinct, so-called atypical, strains of *T. gondii* circulating in the wolverines of the Yukon.

6.6 Conclusions

This is the first report of *T. gondii* in wildlife from the Yukon, and one of few wildlife studies to use a direct detection method in combination with serology, instead of serology alone. Our results support the use of ELISA to determine exposure to *T. gondii* (at least in wolverines), as positive results strongly predicted presence of DNA in the tissues of animals. Overall, prevalence of *T. gondii* in wolverines in the Yukon was lower than that reported in other wild carnivores elsewhere from northern Canada, which suggests lower amounts of the parasite circulating in the northwestern North America. To determine the source of infection for wolverine, we suggest conducting further studies in potential definitive hosts (lynx), as well as game animals including moose, snowshoe hare and geese. Wolverines can act as sentinel species for foodborne parasites such as *T. gondii* for monitoring future changes in parasite transmission. Finally, future studies are needed to determine the status of viable parasite in tissues, and genotypes of *T. gondii* circulating in the wolverines of the Yukon.

6.7 Acknowledgements

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CHAPTER 7 : DISCUSSION AND CONCLUSION

7.1 On the tip of their tongues: predilection site for *Trichinella* spp. in wolverine

Trichinella spp. infections in wildlife are important due to their zoonotic nature, and become more significant in regions where people consume raw game or meat prepared with traditional methods. To avoid the potential risk of spillover between domestic and sylvatic cycles and to measure potential risk to public health [221], monitoring of wildlife for *Trichinella* spp. is important. We selected wolverines as a sentinel species to determine the status of *Trichinella* (prevalence, intensity and genotypes) in northwestern Canada. In surveillance programs, the selection of appropriate sampling sites plays a crucial role due to unknown distribution of species of *Trichinella* in wildlife hosts. We compared tongue and diaphragm from wolverines (Chapter 2) naturally infected with *Trichinella* spp. Our results indicated that tongues had higher infection levels than diaphragms. This information is a new addition to the literature on a handful of predilection studies performed in wildlife, and would be helpful for biologists, wildlife veterinarians, and researchers. In comparison to diaphragm, the tongue is easier to collect. However, collecting tongues could place the sample collector at risk of exposure to saliva containing rabies. Our findings indicate that either tissue is a good sample for testing for trichinellosis in wolverine. Further work is needed to determine if other muscles (e.g leg, eye, or other muscles) could act as better sample sites for detecting this parasite in wolverines.

7.2 Wolverines eat everything, and are great sentinels for *Trichinella* spp., especially T6

Wolverines had high prevalence, infection intensity, and diversity of *Trichinella* spp. - I report the presence of four known species/genotypes of *Trichinella* in wolverines from northwestern Canada. *Trichinella* T6 and *T. nativa*, freeze resistant species of *Trichinella*, were commonly seen, and co-infections were observed. In addition, we also documented single cases of *T. spiralis* and *T. pseudospiralis* in the wolverines. Therefore, wolverines can host four known species of *Trichinella* (Chapter 4, appendices A and B). Previously, four *Trichinella* species (*Trichinella* T6, *T. nativa*, *T. murrelli* and *T. pseudospiralis*) have only been reported in cougars in British Columbia, Canada [16]. In addition to unidentified host mediated factors, scavenging behaviour and wide home range could be the factors responsible for exposure to this wide range of *Trichinella* species in wolverine [16, 52, 67]. Dominance of the sylvatic species of *Trichinella*

(*Trichinella* T6 was predominant) and only a single case of *T. spiralis* indicates primarily a sylvatic cycle of trichinellosis in the Yukon. Further studies are required to determine if wolverines are reservoirs for *T. spiralis* and/or *T. pseudospiralis*, or if these cases simply represent spillover from anthropogenic movements of pigs, or infected meat, other wildlife hosts such as migratory birds [222]. Next steps would be to conduct microsatellite analysis on both T6 and *T. nativa* isolates from different geographical locations of the Yukon to determine if *Trichinella* T6 can be considered as a distinct species.

7.3 And then there were five – a new species of *Trichinella* in wolverine

Previously, there were 12 known species/genotypes of *Trichinella*; we discovered a previously unrecognized cryptic species of *Trichinella* (Chapter 5). This species of *Trichinella* (designated as T13) along with *Trichinella* T6, and *T. nativa* occur in sympatry geographically (Yukon, Northwest Territories) and the same hosts (wolverine). Therefore, wolverines can act as reservoir hosts for five species of *Trichinella*; *Trichinella* T6, *T. nativa* (described in chapter 3), *T. spiralis* (Appendix A), *T. pseudospiralis* (Appendix B) and *Trichinella* T13. Moreover, T13 is the sister of an assemblage or subclade of encapsulated species of *Trichinella*. It is not closely related to T6 or *T. nativa* nor other species in North America. We explore hypotheses for the origin and distribution of T13 relative to an assemblage of encapsulated species found in North America; this warrants further studies on Alaskan and Russian isolates of *Trichinella*. Infections of T13 were detected only in wolverines, and prevalence was higher in wolverines from the Yukon than from Northwest Territories (although sample size from the Yukon was much higher).

Multiplex PCR commonly used in the identification of *Trichinella* isolates misidentified T13 as *T. nativa*, highlighting the need for sequencing, ideally of multiple loci or larger regions of the genome, to detect cryptic species and to accurately characterize diversity. Another significant finding was freeze resistant behaviour of the new species of *Trichinella*. Almost all of the positive animals had tightly coiled larvae, which morphologically indicate viability; however, only one positive animal had one motile larva. Further mouse inoculation tests are required to confirm the viability and pathogenicity of T13 relative to other species of *Trichinella*.

7.4 Wolverines have a lot of *Toxoplasma gondii* in their heart – “Killing two birds with one stone”

In this thesis, I found that heart can be used for both serology and PCR (“killing two birds with one stone”) for detection of exposure to and infection with another important foodborne parasite, *Toxoplasma gondii*. We found that heart was a better sampling site than brain for

detection of *T. gondii* DNA in wolverines due to the following findings: (1) a higher parasite burden, (2) more practical sampling site than brain, and (3) concurrent availability of heart fluid that can be used for comparative serological testing. We selected heart and brain as they were considered as common predilection sites of *T. gondii*, but there could be other preference sites for the parasite; therefore, further work should include MC-qPCR on other tissues (such as skeletal muscle) to determine the predilection sites for *T. gondii* in wolverine. As serum from dead wild animals is usually unavailable, heart fluid can be a good candidate matrix to detect anti-*T. gondii* antibodies in wolverines using ELISA, IFAT and MAT, but cut-off values and false positives (low specificity) need to be further investigated for MAT. We suggest evaluating the performance of commercially available kits designed to detect *T. gondii* antibodies in domestic animals before their usage in wildlife. As controls from experimentally infected animals are generally lacking for wildlife, validation is seldom possible. Best practices should include multiple assays and sensitive tests to determine infection status of naturally infected animals; for example, MC-qPCR, when bioassay is not feasible (frozen tissues, large scale studies) or desirable (animal research ethics). In this thesis, I found good agreement between two serological tests and one direct detection method for *T. gondii* in wolverine, inspiring confidence in these assays for future use in terrestrial carnivores.

7.5 *Toxoplasma gondii* is present in terrestrial wildlife in northwestern Canada

Our study is the first report of DNA of *T. gondii* in wildlife in the Yukon, which suggests that wildlife in north-western Canada are exposed to *T. gondii*; however, prevalence was lower than expected, especially given high prevalence of *Trichinella* (another food-borne parasite) in the same wolverine, and high prevalence of *Toxoplasma* reported in wolverine in the adjacent Northwest Territories and Nunavut. Wolverines are not game animals, but they are harvested for their fur, and skinning of fur animals has been reported as a risk factor for *T. gondii* exposure in people [92]. We recommend that fur trappers use caution when handling wildlife carcasses, and avoiding leaving carcasses in the field after harvesting to prevent scavenging and perpetuation of the life cycle of this potential foodborne zoonosis. *Toxoplasma gondii* has the potential to influence health and reproductive success of mustelids, and I suggest future studies to determine if *T. gondii* could affect the health and reproduction of wolverines. It will also be important to determine the genetic diversity of *T. gondii* in wolverine to determine if they are primarily infected with clonal types of *T. gondii* common in domestic animals and people in temperate regions, or if there are distinct, so-called atypical, strains of *T. gondii* circulating in the wolverines of the Yukon.

Certainly, my findings with *Trichinella* suggest that there may be unexpected diversity of food borne parasites in wolverine, who justly deserve their scientific name (meaning the glutton). Overall, wolverines can act as sentinel species for foodborne parasites such as *Trichinella* spp. and *T. gondii* for monitoring future changes in parasite transmission.

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Appendix A

In chapter 3, I found high prevalence and larval intensity of *Trichinella* spp. in wolverines in the Yukon. *Trichinella* T6 was the predominant genotype of *Trichinella*, followed by *T. nativa*; mixed infections were also present. Moreover, larvae of *T. spiralis* were present in one wolverine. Canadian market pigs are considered free of *T. spiralis*. This appendix discusses possible origin/transmission routes of this isolate, including the possibility of its circulation in wildlife across an international border.

***Trichinella spiralis*, a reportable parasite in pigs, in a wolverine (*Gulo gulo*) from the Yukon, near the Alaskan Border**

Rajnish Sharma¹, Kelly Konecsni², Peter Thompson³, N Jane Harms⁴, Piia M Kukka⁴, Tom S Jung⁴, Brent Wagner¹, Eric P Hoberg⁵, Benjamin Rosenthal³, Brad Scandrett² and Emily Jenkins¹

1 Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

2 Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada.

3 USDA, Agricultural Research Service, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705, USA.

4 Environment, Yukon Government, P.O. Box 2703, 10 Burns Road, Whitehorse, YT, Canada, Y1A 2C6.

5 Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131-0001

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Abstract

Five *Trichinella* species (*T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6) have been documented in domestic and wild animals in Canada. *Trichinella spiralis* has been eradicated from commercial confinement-raised pigs and is immediately reportable to animal health authorities in Canada. Here, we report *T. spiralis* in a 1 year old male wolverine (*Gulo gulo*) from the Canadian North. Larvae recovered from tongue and diaphragm of the infected animal were identified as *T. spiralis* by multiplex polymerase chain reaction. Identification was further confirmed by sequencing of the *Nad 5* mitochondrial DNA gene. The infected wolverine was harvested from the Klondike region located on the border of the Yukon and Alaska. This is the first recorded occurrence of *T. spiralis* in the Canadian sub-arctic region. There is very little domestic swine production in the Yukon or neighboring regions of Alaska. Therefore, it is theorized this young wolverine potentially scavenged imported meat, backyard pigs raised for personal consumption, feral pigs or wild boar, or migratory wildlife infected with the parasite. This finding emphasizes the need for transboundary surveillance in wildlife near international borders, and cross border cooperation to mitigate spread of pathogens that can impact public or animal health.

Introduction:

Trichinellosis is an important food borne disease caused by nematodes of the genus *Trichinella* (Gottstein et al., 2009). People become infected through consumption of larvae in raw/improperly cooked meat, and manifestations of trichinellosis may include fever, weakness, myalgia, diarrhea, facial edema, and even fatal myocarditis (Gottstein et al., 2009). The *Trichinella* genus has 12 taxa grouped into two clades: encapsulated (*T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *Trichinella* T6, *T. nelsoni*, *Trichinella* T8, *Trichinella* T9, and *T. patagoniensis*) and non-encapsulated (*T. pseudospiralis*, *T. papuae* and *T. zimbabwensis*) (Pozio and Zarlenga, 2013). Only five species or genotypes of *Trichinella* have been reported in wildlife in Canada: *T. nativa*, *Trichinella* T6, *T. murrelli*, *T. pseudospiralis* and *T. spiralis*. These are present in a wide range of carnivores and omnivores, but only *T. pseudospiralis* has been reported in birds (Gajadhar and Forbes, 2010; Jenkins et al., 2013; Pozio et al., 2009a).

T. spiralis has been reported sporadically in wildlife in Canada (Appleyard et al., 1998) and in both experimentally and naturally infected wild animals elsewhere (Appleyard et al., 1998; Kapel, 2000; Kapel et al., 2005; Pozio et al., 2009b). In contrast to all other taxa in the genus, *T. spiralis* has a pastoral cycle and is generally associated with pigs and rats while the others are associated with sylvatic cycles in wildlife. However, similar to commercial swine raised under controlled management conditions in other industrialized countries, Canadian market pigs are considered free of *T. spiralis* (Appleyard et al., 2002; Appleyard and Gajadhar, 2000; Gajadhar et al., 1997; Gajadhar and Forbes, 2010). The last reported autochthonous *T. spiralis* infection in commercial pigs occurred in Nova Scotia in 1996 (Gajadhar et al., 1997), although a case in pigs raised for personal consumption in Ontario was recently reported (Newman, 2014). Even the suspicion of *T. spiralis* must be immediately reported to animal health authorities in Canada.

In Canada, most human cases of trichinellosis are now linked to consumption of meat from wild animals (black bear, grizzly bear, walrus) (Dalcin et al., 2017; Houze et al., 2009; Schellenberg et al., 2003), generally with freeze-tolerant *T. nativa* identified as the causal agent in those outbreaks where molecular characterization of larvae was performed. Although wild carnivores [wolves (*Canis lupus*), wolverines (*Gulo gulo*), foxes (*Vulpus* spp.)] are not typically consumed by humans, they are important reservoirs of *Trichinella* and thus can act as indicator host species in surveillance programs (Dick, 2001). Wolverines are an economically important species in northern Canada because of their valuable fur. Their predatory and scavenging lifestyle and large home range predispose wolverines to exposure to foodborne parasites such as

Trichinella. Through surveillance in wolverines for *Trichinella* spp., we serendipitously detected one wolverine infected with *T. spiralis* in subarctic Canada, an unexpected finding given the absence of commercial swine production in the North and the eradication of this parasite in pigs in Canada. In a study, *T. spiralis* has not been detected in wild animals when the local pigs are free of this parasite (Hill et al., 2010). We discuss possible origin/transmission routes of this isolate, including the possibility of its circulation in wildlife across an international border.

Material and methods:

Collection and transportation of samples: In June 2016, we collected organs and tissues from carcasses of wolverines harvested by trappers/hunters as part of the 2015-16 fur harvest in the Yukon. Tongues and diaphragms were stored at -20°C and shipped to the Zoonotic Parasite Research Unit (ZPRU), Department of Veterinary Microbiology, University of Saskatchewan.

Recovery of larvae: Larvae were recovered from tongue tissue using the double separatory funnel digestion method (Forbes and Gajadhar, 1999). Briefly, 10 g of lean muscle was digested in 1% pepsin HCl solution, followed by sequential sedimentation in two separatory funnels. Sediment fluid was collected in a petri dish and larvae were observed under the dissection microscope (10-16X power) and classified into live (tightly and loosely coiled) and dead (comma shaped) larvae. Larvae per gram (LPG) was calculated by dividing total number of larvae recovered by weight of digested muscle. Motility of larvae was checked by incubating the Petri dish at 37°C for 30 min, and observed under microscope for motility. Percent motility was calculated by counting number of motile larvae out of total larvae observed in the Petri dish. Larvae were stored in 1X PCR buffer at -20°C till further use. Diaphragm tissue and larvae recovered from tongue tissue were also supplied to Centre for Food-borne and Animal Parasitology (CFAP), Canadian Food Inspection Agency (CFIA) for testing and confirmation.

Molecular characterization: DNA was extracted by Proteinase K method; briefly, five individual larvae and a pool of 10 larvae were subjected to 90°C for 10 min followed by cooling on ice for 5 min. Generally, we select only tightly coiled or motile larvae (presumed to be alive) for PCR, but because of low live larval counts, we used non-motile larvae (c shaped) from this animal. Proteinase K was added and samples were incubated at 48°C overnight. Samples were incubated at 90°C for 10 min, then centrifuged before storing at -20°C till further analysis. Larvae were identified to species using a multiplex Polymerase Chain Reaction targeting ITS-1, ITS-2 and ESV regions of nuclear DNA (Zarlenga et al., 1999). Larvae of *T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6 were obtained from CFAP as positive controls.

PCR was performed on 25 µl volume of master mix (AmpliTaq Gold 360 master mix, GC enhancer and primers) in a thermocycler (which thermos cycling instrument) with the following conditions: denaturation at 95°C for 10 minutes, followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 55°C for 15 s, extension at 72°C for 90 s, followed by final extension at 72°C for ten minutes. Species was identified based on the band pattern seen on 2.5% agarose gel when analyzed under UV light using Gel Doc system.

Sequencing: For further confirmation of species identification, as well as to determine its relation with other isolates of *T. spiralis* and other *Trichinella* spp., a fragment of the Nad 5 mtDNA gene was amplified with primers designed using Primer3 tool. Polymerase chain reaction was performed using forward primer JLF 5' ATTGCTATGGCGGCTCCTAC and reverse primer JLR 5' CCGGAACAACCATCACCTGA. Amplification resulted in PCR products of approximately 650 bp, which were purified using ExoSAP-IT as per manufacturer's instructions before sending for sequencing to Macrogen Korea (Macrogen Inc., Seoul, Korea). The nucleotide sequences of Nad 5 were paired and trimmed using Geneious 11.1.5 program and blasted in the ncbi BLAST to confirm the *Trichinella* species, and to obtain the nucleotide identity with other isolates. The nucleotide sequences of isolates were deposited into the GenBank database. Multiple sequence alignments were carried out using Geneious 11.1.5 program, followed by manual optimization and comparison against the sequences of *T. spiralis* and other species of *Trichinella* available in GenBank. Phylogenetic analysis was performed using Geneious 11.1.5. Neighbor-joining phylogenetic trees were built using different genetic distance models (HKY, Jukes-Cantor, Tamura-Nei).

Results:

The infected wolverine was a 1 year old male originating from the Klondike Plateau region of the Yukon. Its body condition index score was 20.99. Digestion of tongue resulted in 69 (9 tightly coiled, 7 loosely coiled and 53 comma-shaped dead larvae at ZPRU) and digestion of diaphragm recovered 16 (4 loosely coiled and 12 dead larvae at CFAP). None of the recovered larvae were motile. Intensity of *T. spiralis* infection in tongue and diaphragm muscles were 6.9 and 1.1 LPG, respectively. The overall mean larval intensity of *T. spiralis* in wolverine was 3.4 LPG of muscle tissue (tongue and diaphragm together). Multiplex PCR by both ZPRU and CFAP resulted in amplified DNA with a band size of 127 bp from single as well as pooled larvae and indicated a single species infection of *T. spiralis*.

Amplification of the Nad 5 gene resulted in a 650 bp product. Total length of alignment was determined by the length of the shortest entry in GenBank (582 bp). Analysis of DNA sequencing by BLAST confirmed the isolate as *T. spiralis*, with a nucleotide identity of 100% with those of *T. spiralis* isolates from Genbank (Accessions Nos. AF293969, GU 336314 and KM 357422). Similar topologies of phylogenetic trees were seen with all models (data not shown) and therefore only the neighbor joining phylogenetic tree based on the HKY model is presented here (Fig 1). Our *T. spiralis* isolate clustered with other *T. spiralis* isolates, and was separate from other species of *Trichinella* obtained from Genbank [*T. nativa*, Accession No: NC025752; *T. britovi*, NC025750; *T. pseudospiralis*, KM357409; *T. murrelli*, NC025751; *Trichinella* T6, KM357418; *T. nelsoni*, NC025753; *Trichinella* 8, KM357419; *Trichinella* 9, KM357420; *T. zimbabwensis*, NC025755; *T. papuae*, NC025754; *T. patagoniensis*, NC357412]

Discussion:

While conducting surveillance for presumably northern-adapted, sylvatic species of *Trichinella* (*T. nativa*, T6), we detected larvae of *T. spiralis* from a wolverine in the Canadian North. This was surprising because this is primarily a parasite of pigs, which are uncommon in the Canadian North, and this parasite has been eradicated from commercial pigs in Canada. As well, *T. spiralis* is not freeze tolerant and thus not likely to establish in extreme northern climates. However, this is not the first report of *T. spiralis* in a wolverine, which has been documented previously from Yakutia, Russia (Odoevskaya and Spiridonov, 2014). Studies in wolverines from the Yukon and neighboring provinces (NT, British Columbia, Alaska and Nunavut) showed the presence of *Trichinella* spp. larvae, but *T. nativa* and *Trichinella* T6 were the only species identified in samples from the YT, NT and Nunavut (Gajadhar and Forbes, 2010; Reichard et al., 2008; Sharma et al., 2018).

The first verified report of *T. spiralis* in wild animals of Canada was documented in red foxes (*Vulpus vulpus*) and coyotes (*Canis latrans*) originating from Prince Edward Island (Appleyard et al., 1998). Thus, the present case represents the second documented report of *T. spiralis* in Canadian wildlife. In an extensive survey of wildlife of Canada, no *T. spiralis* infection was observed; however infections with *T. nativa*, *Trichinella* T6, *T. murrelli* and *T. pseudospiralis* were reported (Gajadhar and Forbes, 2010). Our work suggests that further surveillance is needed to determine if *T. spiralis* is circulating among Canadian wildlife, even in regions far removed from commercial swine production.

Trichinella spiralis is cosmopolitan in distribution and has been reported from more than 30 countries, but has not been reported from the Arctic, which could be attributed to freeze susceptibility of larvae, preventing transmission via frozen carcasses (Pozio et al., 2009a). The Yukon is one of three northern territories of Canada, and is bordered by Alaska in the west, Northwest Territories in the east and British Columbia in the south. The infected wolverine came from the very southwestern part of the Klondike region, about 50 km southeast of the community of Beaver Creek. Due to the large home range (100-900 km²) of wolverines (Banci, 1994; Mulders, 2001), this finding of *T. spiralis* far north of its known distribution could be a result of migration from bordering Canadian provinces and territories, or Alaska. Wolverines, especially young males, may move great distances from their natal grounds, so it could have travelled anywhere within a several hundred-km radius. Our finding emphasizes the need for transboundary surveillance in wildlife, and cross border cooperation to regulate importation of animals and animal products across domestic and international borders.

Source of infection/transmission: Predation, cannibalism and scavenging are important routes of transmission of *Trichinella* larvae among wildlife (Mukaratirwa et al., 2013; Mukaratirwa et al., 2017). The wolverine is an omnivorous animal and its diet consists of carrion, small mammals, birds and vegetation (Pasitschniak-Arts and Larivière, 1995). This particular wolverine could have acquired *T. spiralis* infection from scavenging local domestic pigs or wild boars. There is at least one commercial farm in Dawson that has pigs, and probably a few "hobby" farms with pigs in the Yukon and nearby regions of Alaska, but not in the Beaver Creek area. In Yukon, there could also be a possibility of contact between wildlife and wild boars (<https://www.cbc.ca/news/canada/north/wild-boars-yukon-mendenhall-1.4741779>). Wild boars are considered as a primary reservoir host in the sylvatic cycle of *T. spiralis* (Kapel, 2001; Pozio et al., 2009a). Countries previously endemic and currently free for *T. spiralis* in their commercial swine still have high prevalence in wild boars (Pozio et al., 2009b). No data on the current status of *Trichinella* infection in domestic pigs and wild boars in the Yukon and neighboring territories/provinces (NT, BC and Alaska) are available; however, a study conducted in Quebec, Ontario, Manitoba and Saskatchewan in 1993-94 showed no *Trichinella* infection in wild boar (Gajadhar et al., 1997). Another possible source of infection could be consumption of illegally imported infected game/pig meat by a visitor from an endemic country, in garbage or possibly used as bait by trappers (Garbarino et al., 2017). It seems unlikely that this wolverine became infected through consumption of local rodents; although *Trichinella* has been reported in Brown

Rat (*Rattus norvegicus*), Ground Squirrel (*Citellus undulates*), Red-backed Vole (*Myodes/Clethrionomys rutilus*), Red Squirrel (*Tamiascurus hudsonicus*) and Brown Lemming (*Lemmus trimucronatus*) of Alaska (Rausch et al., 1956; Schiller, 1952), these would more likely be *T. murelli*, *T. nativa*, or T6. While migratory birds may well account for the recent detection of *T. pseudospiralis* in wolverine in the Canadian North (Appendix B), *T. spiralis* has not been reported in birds. Further surveillance, including molecular characterization of dead larvae from frozen muscle tissue of wildlife origin, as well as methodologies targeting more genes (e.g. microsatellite analysis) could be helpful to detect and compare *T. spiralis* isolates from wildlife from different geographic locations.

Worldwide, *T. spiralis* is the most common species of *Trichinella* implicated in human trichinellosis, and most cases occur after consumption of raw/improperly cooked meat of infected pigs and wild boar. In Canada, the vast majority of human trichinellosis cases are due to consumption of larvae of *T. nativa* (T2) in wildlife such as bear and walrus. The last case of human trichinellosis due to *T. spiralis* in Canada was reported in a three-year-old girl in 2013 from Ontario. *Trichinella spiralis* larvae were identified in pork from domestic pigs raised for personal consumption, which were fed a non-commercial diet that included carcasses of raccoon, duck, and wild rabbits (Newman, 2014). Wild animals can play a role in spillover of *Trichinella* to domestic pigs and vice versa. However, they are unlikely to become established reservoirs of *T. spiralis* in the absence of infected pigs and/or rats (Hill et al., 2010). In Canada monitoring and surveillance of pigs, horses and wildlife for *Trichinella* infection is conducted by the Canadian Food Inspection Agency. The CFIA Centre for Food-borne and Animal Parasitology in Saskatoon is an OIE (World Organization for Animal Health) reference laboratory for *Trichinella* and confirmed positive results by digestion assay and multiplex PCR in the current study.

Wolverines are harvested for their fur and are not consumed for food. Detection of *T. spiralis* in wolverine does not pose a direct food safety risk to people, but raises the possibility of circulation of *T. spiralis* in local domestic and/or game animals, underscoring the importance of cooking of game meat or pork before consumption in order to kill all *Trichinella* species (*T. nativa*, *Trichinella* T6 as well as *T. spiralis*). Freezing also inactivates larvae of *T. spiralis*, but is not reliable inactivation method for freeze tolerant sylvatic *T. nativa* and T6.

Conclusion:

This finding of *T. spiralis* in a wolverine, a reportable parasite eradicated from commercial swine in Canada, supports the need for enhanced surveillance in wildlife, domestic and wild pigs

of the Yukon and Alaska. The detection of *T. spiralis* beyond the known boundaries of its occurrence opens new questions regarding how or if this *Trichinella* species can persist in northern regions.

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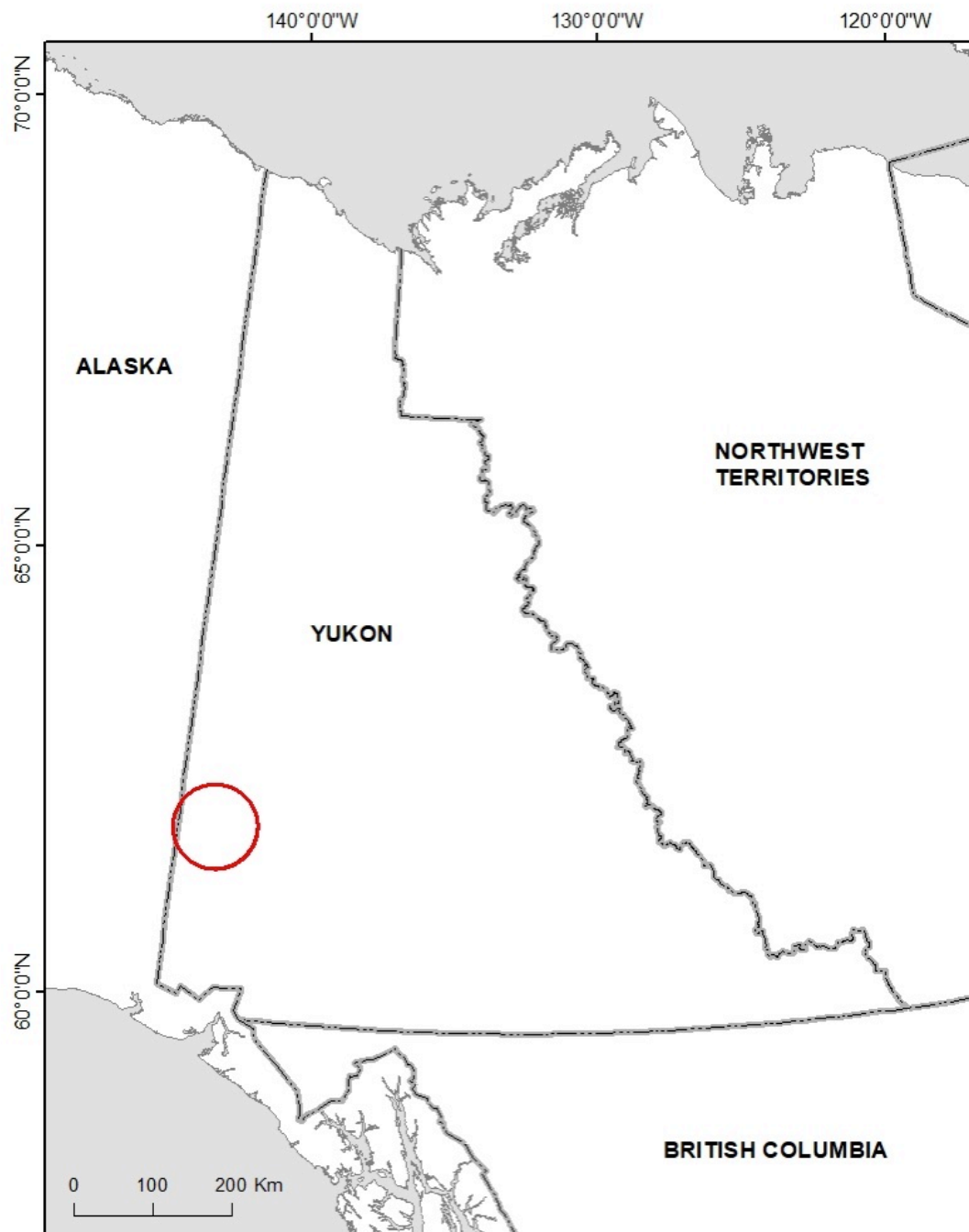


Figure A-1 An outline map of northern North America showing geographic location of wolverine infected with *Trichinella spiralis*. Red circle indicates maximum host range of the infected wolverine.

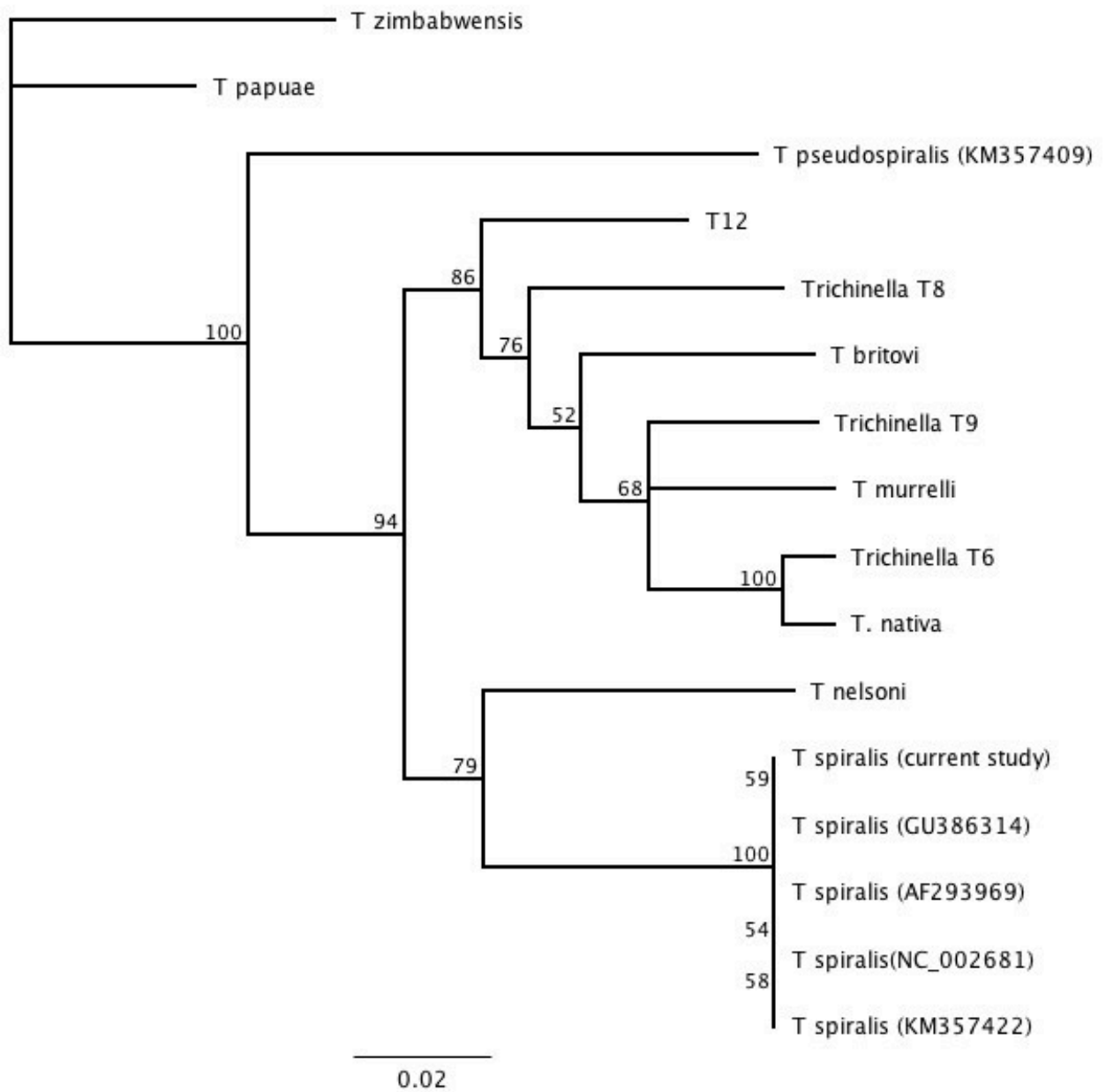


Figure A-2 Phylogenetic tree showing relationship of current *Trichinella spiralis* isolate with other isolates

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Appendix B:

In chapter 3 and appendix A, I reported that there were three species of *Trichinella* found in wolverines: *T. nativa*, *Trichinella* T6, and *T. spiralis*. Five *Trichinella* species *T. spiralis*, *T. nativa*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6 have been documented in domestic and wild animals in Canada. The only previous report of *T. pseudospiralis* in Canada was from a mountain lion (*Puma concolor*) from Vancouver Island in the southwestern region of the country in 2003. This appendix discusses the discovery of *T. pseudospiralis* in a wolverine from northern Canada, and the epidemiological/phylogenetic associations of this isolate of *T. pseudospiralis*.

***Trichinella pseudospiralis* in a wolverine (*Gulo gulo*) from the Canadian north**

Rajnish Sharma¹, Peter Thompson², Brett Elkin³, Robert Mulders³, Marsha Branigan⁴, Jodie Pongracz⁴, Brent Wagner¹, Brad Scandrett⁵, Eric Hoberg⁶, Benjamin Rosenthal² and Emily Jenkins¹

1 Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4

2 USDA, Agricultural Research Service, Animal Parasitic Diseases Laboratory, Beltsville Agricultural Research Center, 10300 Baltimore Avenue, Beltsville, MD 20705, USA.

3 Environment and Natural Resources, Government of the Northwest Territories, 600, 5102-50th Avenue, Yellowknife, NT, Canada, X1A 3S8

4 Environment and Natural Resources, Government of the Northwest Territories, P.O. Box 2749, Shell Lake, Inuvik, NT, Canada, X0E 0T0

5 Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon Laboratory, 116 Veterinary Road, Saskatoon, Saskatchewan, Canada.

6 Museum of Southwestern Biology and Department of Biology, University of New Mexico, Albuquerque, New Mexico 87131-0001

Conceived and designed the experiments: RS, PT, RB, EJJ; Provided mentorship, logistics and supplies: PT, EPH, BS, BE, RM, MB, JP, BW, RB, EJJ; Collected samples and supplied specimens: RS, BS, BE, RM, MB, JP. Performed the experiments: RS, BW; Analyzed the data: RS, PT, RB, EJJ; Wrote the manuscript: RS; Approved the final version of the manuscript: RS, PT, EPH, BS, BE, RM, MB, JP, BW, RB, EJJ

Corresponding author: ras863@mail.usask.ca

Status of manuscript: In preparation

Abstract

Species of *Trichinella* are a globally distributed assemblage of nematodes, often with distinct host ranges, which include people, domestic, and wild animals. *Trichinella* spp. are important in northern Canada, where dietary habits of people and methods of meat preparation (drying, smoking, fermenting as well as raw) increase the risk posed by these foodborne zoonotic parasites. Outbreaks in the north are generally attributed to *T. nativa* (T2) or the T6 genotype; however, genetic characterization is seldom performed, precluding definitive diagnosis. We report the discovery of *Trichinella pseudospiralis* (T4), a non-encapsulated species, in a wolverine (*Gulo gulo*) from the Northwest Territories. This parasite has been previously reported elsewhere from both mammals and carnivorous birds, but our findings represent new host and geographic records for *T. pseudospiralis* in northern Canada. Multiplex PCR and sequencing of fragments of Cytochrome Oxidase Subunit I (COI) and D3 rDNA confirmed the identification. Phylogenetic analysis linked this isolate with others derived from the Palearctic or Neotropical regions. We suggest that migratory birds might have played a role in the dispersal of this pathogen 1000s of km to northwestern Canada. Wolverines are not typically consumed by humans, and thus should not pose a direct food safety risk for trichinellosis. However, the current finding suggests that they may serve as an indicator of a broader distribution for *T. pseudospiralis*. Along with infection risk already recognized for *T. nativa* and *Trichinella* T6, our observations emphasize the need for further studies using molecular diagnostics to clarify if this is a solitary case or if *T. pseudospiralis* circulates in the Canadian Arctic.

Introduction

Among the 24 most significant foodborne parasitic diseases listed by the World Health Organization/United Nations Food and Agriculture Organization, *Trichinella spiralis* globally ranks seventh, with other *Trichinella* spp. ranked as 16th (FAO/WHO, 2014). In a systematic review, 65 818 human cases of trichinellosis were reported worldwide from 1986-2009 (Murrell and Pozio, 2011). From a public health perspective, species of *Trichinella* (largely *T. nativa* and *Trichinella* T6) were ranked third among nine zoonotic parasites in northern North America based on an evidence-based qualitative risk analysis (Jenkins et al., 2013).

People or other mammals and birds contract *Trichinella* spp. infection after consuming meat infected with larvae of these parasites (Gottstein et al., 2009; McIntyre et al., 2007; Serhir et al., 2001). In the stomach of an exposed host, larvae are released when tissue cysts are exposed to stomach acids. These larvae penetrate the small intestine and mature into adult worms within a few days of infection. Males and females copulate and after 5 days post infection, females start releasing newborn larvae, which travel via blood and lymph to predilection sites in the skeletal musculature. For encapsulated species of *Trichinella* such as *T. spiralis*, larvae encyst inside muscle cells, whereas in other species such as *T. pseudospiralis*, larvae remain unencapsulated. Clinical manifestations are rarely recognized in animals, but human patients may display symptoms including headache, fever, abdominal pain, diarrhea, myalgia, eyelid/facial edema, and even mortality due to cardiac manifestations, depending on infective dose and immune status (Gottstein et al., 2009). *Trichinella pseudospiralis* causes clinical manifestations similar to those caused by *T. spiralis* except with a more prolonged myopathy (Jongwutiwes et al., 1998). Diagnosing which species of *Trichinella* is responsible for human outbreaks is desirable, but rarely performed as it necessitates muscle biopsy and comparative molecular analyses. A broad understanding of the species/genotypes of *Trichinella* circulating in domestic and wild animals can aid understanding of transmission pathways, routes of exposure and in developing possible management goals, because species differ in important characteristics, such as host affinities and tolerance to freezing.

All species and genotypes of *Trichinella* have been reported in mammals, whereas *T. papuae* and *T. zimbabwensis* also infect reptilian hosts. *Trichinella pseudospiralis* is the only species in the genus reported in both carnivorous birds and mammals, but the number of reports in mammals exceeds those in avian hosts (Pozio et al., 2009; Zamora et al., 2015). Worldwide, *T. pseudospiralis* has a cosmopolitan distribution and has been reported in 18 mammalian and eight avian species

(Pozio, 2016). In Canada, *T. spiralis* has been eradicated from commercially raised pigs, and has only rarely been reported from wildlife (Gajadhar and Forbes, 2010). In contrast to the domestic cycle, a number of sylvatic species of *Trichinella* exist in Canadian wildlife, and include *T. nativa* (T2), *T. murrelli* (T5), *Trichinella* T6, and *T. pseudospiralis* (Jenkins et al., 2013). *Trichinella nativa* and *Trichinella* T6 are freeze-tolerant, and are the most common species found in wildlife hosts in the arctic and sub-arctic zones of Canada. Both species are commonly found in wolves (*Canis lupus*), wolverines (*Gulo gulo*), bears (*Ursus* spp.), arctic foxes (*Vulpus lagopus*), walruses (*Odobenus rosmarus*), and other carnivorous mammals (Gajadhar and Forbes, 2010; Jenkins et al., 2013; Sharma et al., 2018). Recent outbreaks of trichinellosis in Canada have been almost exclusively linked to consumption of game meat (black bear, grizzly bear, walrus) infected with *T. nativa* (Dalcin et al., 2017; Houze et al., 2009; McIntyre et al., 2007; Schellenberg et al., 2003; Serhir et al., 2001).

The public health importance of trichinellosis in the Canadian north necessitates continuing surveillance for species of *Trichinella* in a diverse assemblage of largely mammalian wildlife species. Wolverines are not commonly consumed, but are commonly harvested for fur, and have high prevalence ($\geq 80\%$) of this parasite due to their high trophic position and scavenging lifestyle (Reichard et al., 2008). Therefore, they make excellent sentinels for this and other food borne parasites. Here, we report the discovery of *T. pseudospiralis* in a wolverine from northern Canada, and discuss the epidemiological/phylogenetic associations of this isolate of *T. pseudospiralis*.

Material and methods

Sample collection and transport:

Wolverine carcasses were submitted to the Department of Environment and Natural Resources, Government of the Northwest Territories as part of the fur harvest during 2005-2012 in NWT, and included 127 animals. Tongues and diaphragms were collected from wolverine carcasses and were stored at -20°C before shipping to the Department of Veterinary Microbiology, Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada.

Sample preparation, digestion and recovery of larvae

Fat and connective tissue were removed from the tongue (or diaphragm, if tongue was not available) of each wolverine, and muscle was cut into 0.5-1.0 cm cubes, mixed and a portion randomly selected to make up to 10 g. Muscle tissues were processed by the pepsin-HCl double separatory funnel digestion method (Forbes and Gajadhar, 1999). *Trichinella* first-stage larvae (L1) were identified based on morphology observed under the stereo-microscope, and counted.

The burden of infection was estimated as larvae per gram (LPG). Larval motility was assessed by incubating a petri plate containing the larvae at 37⁰ C for 30 min. Five individual larvae and a pool of ten larvae were collected in six 0.6 ml tubes containing 1X PCR buffer [10 mM Tris-HCl, pH 8.3, 50 mM HCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin] and stored at -20⁰C until used for molecular analysis. Compression of small tongue samples using a glass compressorium was also performed to determine if larvae in-situ were encapsulated.

Molecular identification and sequencing

Parasite genomic DNA was extracted from 5 individual larvae as well as a pool of 10 larvae using a Proteinase K extraction method (Scandrett et al., 2018). To identify species or genotype of larvae of species of *Trichinella*, primers amplifying internal transcribed spacer regions (ITS 1 and 2) as well as the expansion segment V (ESV) of the large subunit ribosomal DNA (Zarlenga et al., 2001) were used in a multiplex PCR assay. Positive controls of six recognized species of *Trichinella* (*T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. murrelli* and *Trichinella* T6) were provided as larvae by the Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon. Samples were identified based on the banding patterns of amplified products on the 2.5% agarose gel stained with Red Safe (FroggaBio Inc, ON, Canada) and photographed using a Gel Doc system (Alpha Innotech AlphaImager digital imaging system).

To confirm and compare sequence from the current *T. pseudospiralis* isolate with other isolates of different geographical origin (Eurasia, Australia and USA), the D3 domain of nuclear ribosomal DNA (D3 rDNA) and the mitochondrial cytochrome C oxidase subunit I (COI) gene were amplified by PCR using the primer pair 5-ACCCGTCTTGAAACACGGA-3 and 5-GATTAGTCTTTCGCCCCTA-3 and the primer pair 5-GTTTTTTGGGCATCCAGAAGTT-3 and 5-GAAGAAGGTCTAAGGAAGCATTTGA-3, respectively (Gasser et al., 2004; Krivokapich et al., 2015). Amplified segments of 400 bp of the D3 rDNA and 345 bp of the COI gene were purified using ExoSAP-IT as per manufacturer's instructions and sent to Macrogen Korea for sequencing (Macrogen Inc., Seoul, Korea). Consensus sequences for each locus were generated in Geneious 11.1.5 (Biomatters, Ltd., New Zealand) based on forward and reverse Sanger sequences. BLAST searches of the non-redundant nucleotide database at NCBI GenBank were used to confirm the *Trichinella* species diagnosis from the multiplex, and to obtain the nucleotide identity with other isolates. The nucleotide sequences of D3 rDNA and COI were deposited into the GenBank database under Accession Nos. MK333397 and MK333398, respectively. Multiple sequence alignments were carried out using Muscle 3.8.425 multi alignment

program followed by manual optimization and comparison against the COI and D3 rDNA sequences of *T. pseudospiralis* from different geographical locations available in GenBank (Table A-1). Phylogenetic analysis was performed using Geneious 11.1.5 using the Neighbor-joining algorithm reconstructed from distances calculated using the HKY model of nucleotide substitution.

As animals were harvested for purposes other than research, this was considered Category A and exempt from Animal Research ethics review at the University of Saskatchewan. We worked closely with the Government of the Northwest Territories for wildlife research and export permits.

Results

Microscopic examination

The positive animal was a one-year-old male, which had been trapped in the South Slave Region (Latitude 60.83300 N., Longitude -117.20 W.) in 2006. Digestion of tongue and diaphragm resulted in 12 and 44 larvae, respectively. When repeated, digestion of tongue muscle resulted in 17 larvae. Mean LPG in diaphragm (4.4, 44 larvae/10 g) was more than that in tongue (1.45, 29 larvae/20 g). The overall mean larval burden was 2.4 LPG (tongue and diaphragm combined). None of the larvae were motile. We detected only two non-encapsulated *Trichinella* spp. larvae in 40 compressed tongue muscle samples (Fig.B-1).

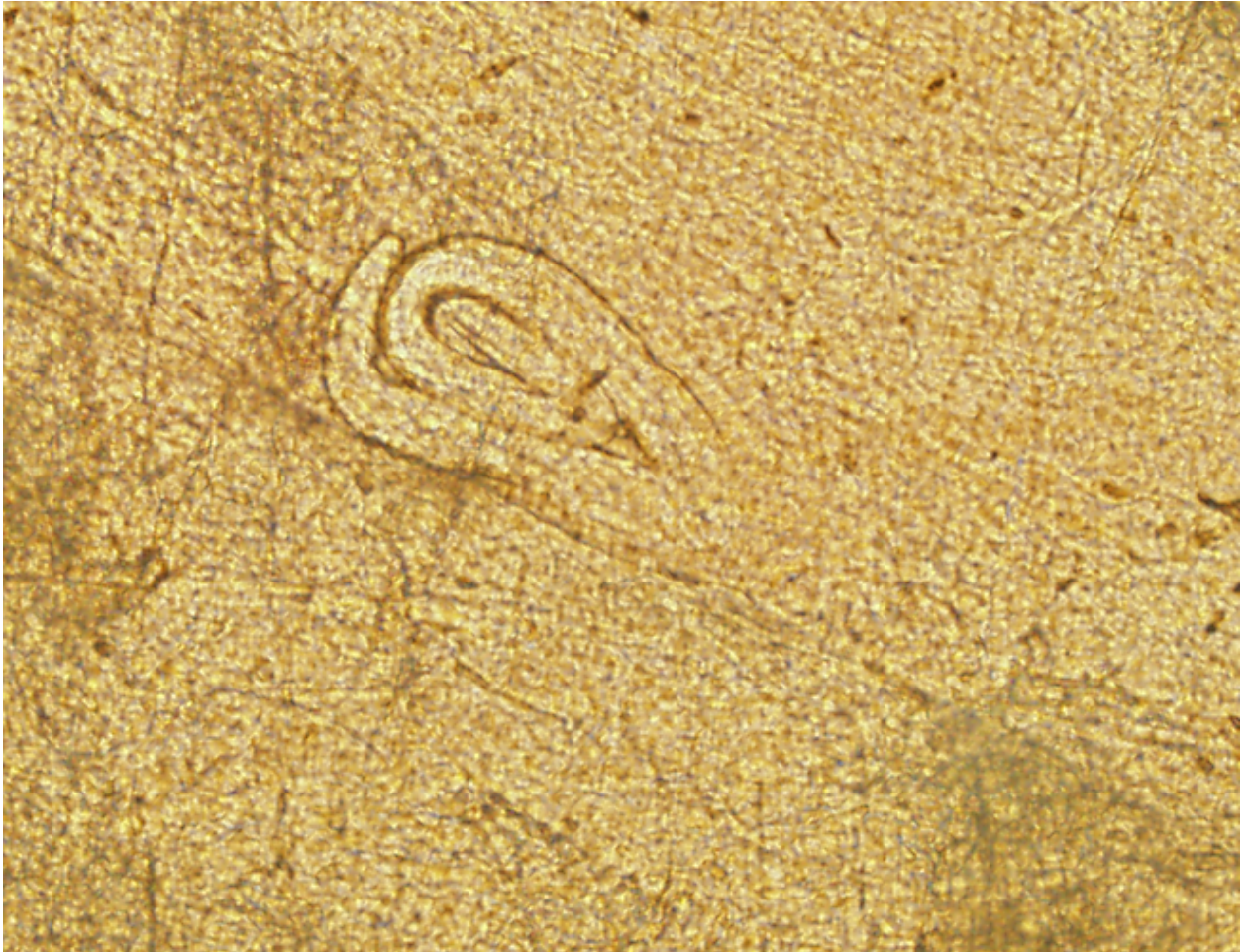


Figure B-1 Photomicrograph of *Trichinella pseudospiralis* larva in compressed tongue muscle of a wolverine (*Gulo gulo*)

Multiplex PCR and DNA sequencing

Multiplex PCR revealed amplicons of the size approximately 310 bp, which corresponds to *T. pseudospiralis*. In order to determine whether this isolate was closely related to any particular previously published isolates, sequence identity was considered and phylogenetic analyses were completed. COI sequences showed a nucleotide identity of 99-100% with those of isolates from Russia, Kazakhstan and Argentina, compared with 97.7% and 94.8-95.1% with those of isolates from Australia and USA, respectively (Table 1). The D3 rDNA sequences showed a nucleotide identity of 100% with those of the isolates from Russia and Argentina, and 99.3 % and 97.8 % with those of isolates from USA and Australia, respectively (Table 1). The phylogenetic inference and tree topology clustered this isolate with those of *T. pseudospiralis* previously documented from Russia and Argentina (Fig B-2).

Table B-1 Comparison of percentage nucleotide identity of current *T. pseudospiralis* isolate with other isolates from different geographical origins

DNA sequence	Isolate Code	Accession number	Geographic origin	Identity (%)	Reference
COI	ISS13	KM357408	Russia (Krasnodar)	100	(Mohandas et al., 2014)
	ISS588	KM357409	Russia (Kamchatka)	100	(Mohandas et al., 2014)
	ISS176	KM357410	Kazakhstan	100	(Mohandas et al., 2014)
		KM063187	South America (Argentina)	100	(Krivokapich et al., 2015)
	ISS13	DQ007893	Russia	99.7	(Zarlenga et al., 2006)
	ISS141	EF601545	Australia	97.7	(Wu et al., 2007)
	ISS1132	EF601544	USA (Texas)	95.1	(Wu et al., 2007)
	ISS470	KM357411	USA (Alabama)	94.8	(Mohandas et al., 2014)
D3 rDNA	ISS13	AJ633056	Russia (Krasnodar)	100	(Gasser et al., 2004)
		KM063188	South America (Argentina)	100	(Krivokapich et al., 2015)
	ISS470	AJ633058	USA (Alabama)	99.3	(Gasser et al., 2004)
	ISS141	AJ633057	Australia	97.8	(Gasser et al., 2004)

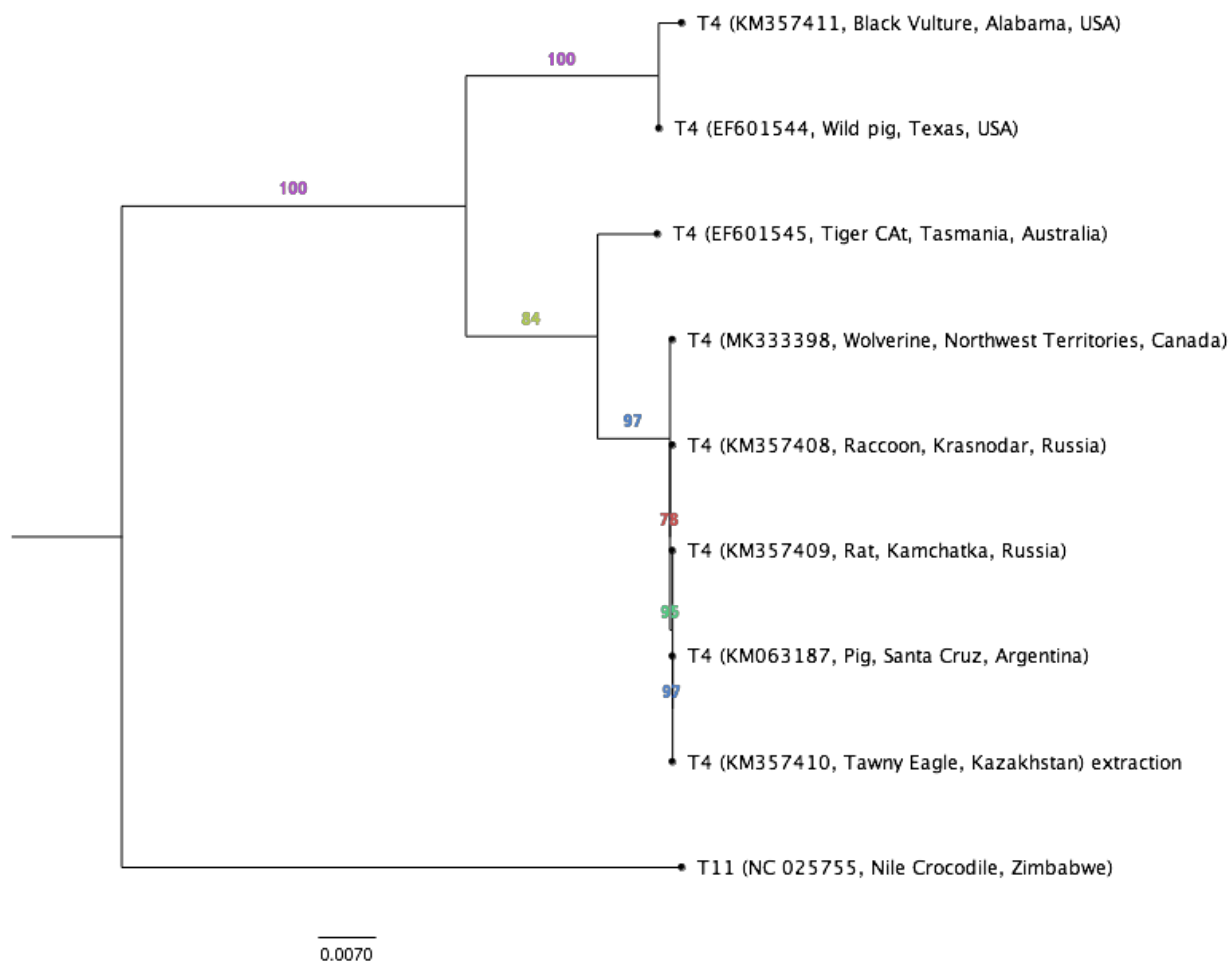


Figure B-2 Phylogenetic tree based on neighbor-joining showing relationship of current *Trichinella pseudospiralis* isolate with other isolates.

Discussion

This is the first report of *T. pseudospiralis* in wolverine and only the third report in any mustelid host; previous reports include badger, *Meles meles* and American mink, *Neovison vison* (Pozio, 2016). In North America, the first three reports were based on histology in a Coopers Hawk (*Accipiter cooperi*) from California, (Wheeldon et al., 1983; Wheeldon et al., 1982), and muscle digests from a Great Horned Owl (*Bubo virginianus*) from Iowa, and a Pomarine Jaeger, *Stercorarius pomarinus* from Alaska, (Rausch et al., 1956; Zimmermann and Hubbard, 1969), but were identified prior to the advent of molecular diagnosis. The first verified North American isolate based on DNA hybridization using a species-specific probe was in a black vulture (*Coragypus atratus*) from Alabama (Lindsay et al., 1995). Based on the presence of unencapsulated, freeze susceptible larvae, the multiplex PCR results, and sequencing of both mitochondrial and nuclear DNA loci, we have confirmed that the isolate obtained from this wolverine is consistent with *T. pseudospiralis*.

In addition to a new host record for *T. pseudospiralis*, this is the first documentation of the parasite on the mainland of Canada, the second report of *T. pseudospiralis* infecting a wild animal from Canada, and the northernmost observation of the parasite from North America (Table B-2, Fig B-3). The only previous report of *T. pseudospiralis* in Canada was from a mountain lion (*Puma concolor*) from Vancouver Island in the southwestern region of the country in 2003 (Gajadhar and Forbes, 2010). Wolverines in North America have varied home ranges from 100 to 900 km² but are known for long-range dispersal (Banci, 1994; Mulders, 2001). Assuming maximum dispersal of 1000 km of the animal under study, this wolverine likely originated within northwestern Canada.

Table B-2 North American and global reports of *T. pseudospiralis* in wildlife

North American reports				
Host	Geographic origin	Year	Number positive	Reference
Black vulture (<i>Coragypus atratus</i>)	Alabama, USA	1995	1	(Lindsay et al., 1995)
Wild boar	Newcastle, Texas, USA	2000	1	(Gamble et al., 2005)
Mountain lion (<i>Puma concolor</i>)	Vancouver Island, Canada	2003	1	(Gajadhar and Forbes, 2010)
Florida panther (<i>Puma concolor coryi</i>)	Florida, USA	2012	18	(Reichard et al., 2015)
Mountain lion (<i>Puma concolor cougar</i>)	Colorado, USA	2014	3	(Reichard et al., 2017)
Wolverine (<i>Gulo gulo</i>)	Northwest Territories, Canada	2006	1	Current study
Global reports				
<i>Rook</i> (<i>Corvus frugilegus</i>)	Chimkent region, Kazakhstan	1975	2	(Shaikenov, 1980)
<i>Tawny eagle</i> (<i>Aquila rapax</i>)	Kazakhstan	1980	1	(Pozio et al., 1992)
<i>Western marsh harrier</i> (<i>Circus aeruginosus</i>)	Tasmania, Australia	1990	1	(Obendorf and Clarke, 1992)
<i>Australian masked owl</i> (<i>Tyto novaehollandiae</i>)	Tasmania, Australia	1990	1	(Obendorf and Clarke, 1992)
<i>Black vulture</i> (<i>Coragypus atratus</i>)	Alabama, USA	1995	1	(Lindsay et al., 1995)

<i>Tawny owl</i> (<i>Strix aluco</i>)	Stockholm, Sweden	1997	1	(Hurníková et al., 2014)
<i>Tawny owl</i> (<i>Strix aluco</i>)	Uppsala, Sweden	2011	1	(Hurníková et al., 2014)
<i>Tawny owl</i> (<i>Strix aluco</i>)	Marche, Italy	1998	1	(Pozio et al., 1999)
<i>Little owl</i> (<i>Athene noctua</i>)	Marche, Italy	1998	1	(Pozio et al., 1999)
<i>Common buzzard</i> (<i>Buteo buteo</i>)	Krasnodar region, Russia	2006	1	(Pozio, 2016)

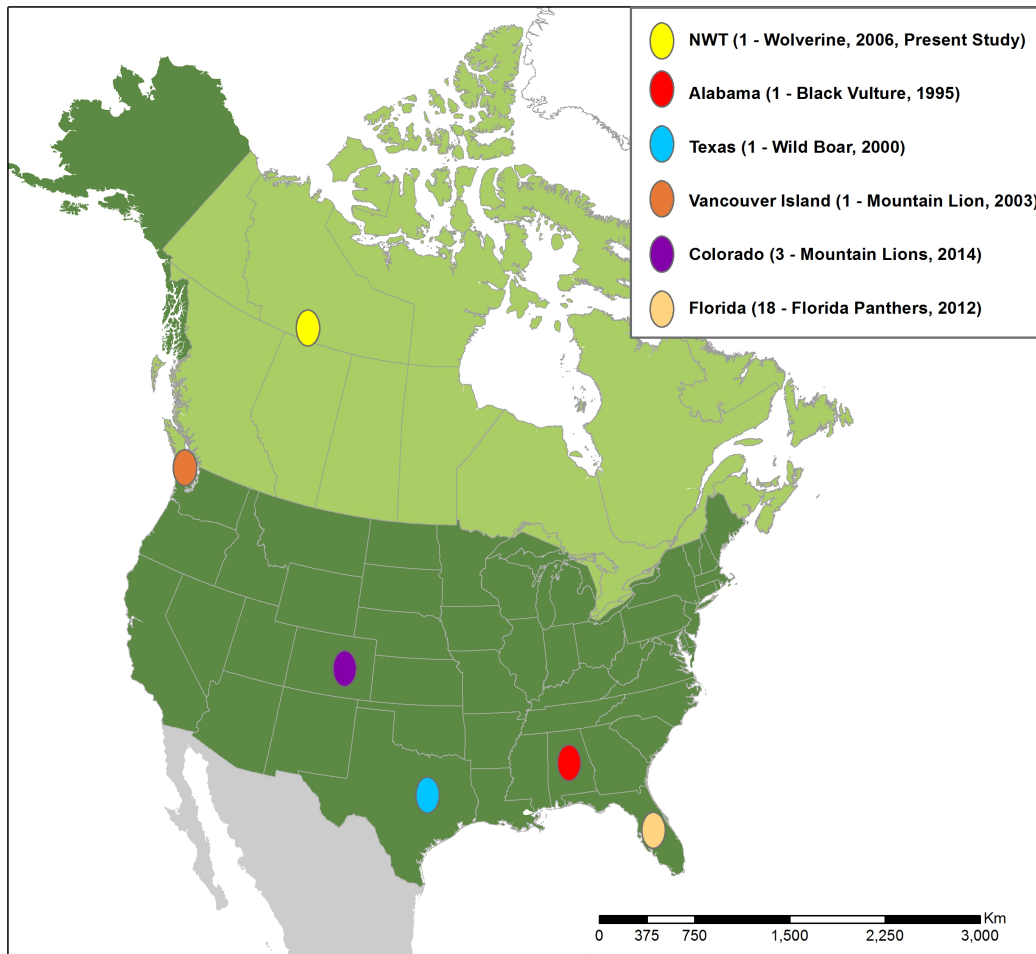


Figure B-3 Geographic locations of this and previously published reports of *Trichinella pseudospiralis* confirmed by multiplex PCR in North America.

We hypothesize that *T. pseudospiralis* in this region could be introduced through migratory birds or mammals. Dietary habits of wolverines vary with season, availability and distribution of prey species and geographical locations. Wolverines are opportunistic foragers, primarily depending on carcasses of caribou (*Rangifer tarandus*), and other carrion and prey in the winter, shifting to vegetation and preying on small mammals, and birds in the summer. (Pasitschniak-Arts and Larivière, 1995). Wild birds, especially raptors and birds such as jaegers that typically feed on small to medium sized mammals (e.g., rodents, shrews, lagomorphs), could spread *T. pseudospiralis* over great distances leading to establishment of new foci of infections in locations previously considered at no or low risk for this parasite (Zamora et al., 2015). Nucleotide sequence analysis placed the isolate discovered in wolverine among populations from the Palearctic or Neotropical region rather than among those from geographically proximate locations in the Nearctic (North America). The closest match (99-100% identity) was with Russian, Kazakhstani and Argentinian isolates, raising the hypothesis of transmission pathways for this isolate linking Eurasia or the Neotropical region to Canada via migratory birds, especially birds of prey. Natural infections of *T. pseudospiralis* have been reported in eight species of birds, primarily raptors as well as corvids such as rook (*Corvus frugilegus*) from Russia and Kazakhstan (Table 2). The first natural infection in an avian species was reported among rooks, a large passerine (Corvidae-*Corvus frugilegus*), from the Chimkent region, Kazakhstan in 1975 (Shaikenov, 1980).

Trichinella pseudospiralis has been reported from mammals more frequently than from birds, but only a limited number of investigations have been directed to avian species. Additionally, the sensitivity of the digestion assay may be limited when applied to the generally smaller muscle samples obtainable from birds. Of 23 previous reports, only one is from a bird in North America (Lindsay et al., 1995). Another possibility could be that this parasite was introduced to the region through a migratory terrestrial mammal covering the considerable distance required to reach this region. As the positive wolverine was located close to small human communities (Fort Providence, Fort Resolution, Hay River, and Enterprise), the wolverine may have acquired its infection by scavenging on discarded meat brought in by people from abroad; however, these are not major hubs for international travel or immigration. Based on this single finding of *T. pseudospiralis*, we cannot assume that it is actively circulating in northern Canadian wildlife; further local sampling of wild mammals and birds seems warranted.

The higher burden of *T. pseudospiralis* larvae in diaphragm versus tongue of this wolverine is consistent with a report in foxes experimentally infected with other species of *Trichinella* (Kapel et al., 2005). In contrast, larvae of *T. nativa* and *Trichinella* T6 had a higher intensity of infection in tongue vs diaphragm in wolverine (Sharma et al., 2018). We did not detect any encapsulated larvae of species of *Trichinella* spp. on examination of compression of tongue from this wolverine, nor were any other species of *Trichinella* (such as T2 or T6) detected on multiplex PCR. In spite of the fact that we used a highly sensitive digestion method for recovery of species of *Trichinella* spp. (Forbes et al., 2003), we found only one positive wolverine (of 131) with *T. pseudospiralis*, whereas prevalence of *T. nativa* and *Trichinella* T6 (Sharma et al, pers. Commun.) was higher.

The digestion assay is validated for the detection of live larvae in fresh samples; therefore, using frozen samples (as the situation here) may have reduced overall sensitivity, especially for freeze susceptible species of *Trichinella* such as *T. pseudospiralis*. The tongue and diaphragm of wolverines tested were kept frozen at -20°C prior to processing, and underwent two cycles of freeze-thaw. Monitoring studies for *Trichinella* based on freshly harvested wildlife carcasses might reveal a higher prevalence of freeze-susceptible species such as *T. pseudospiralis* and *T. spiralis* than previously suspected.

Reports of *T. pseudospiralis* in harvested wild animals from North America indicates that this potential zoonosis is circulating and poses a potential risk to public health. Human outbreaks in North America have not been attributed to *T. pseudospiralis*; however, definitive identification is rarely performed on human isolates (Dalcin et al., 2017; Gamble et al., 2005; Houze et al., 2009; McIntyre et al., 2007; Reichard et al., 2017; Serhir et al., 2001). Wolverines are not harvested for food but rather for fur; thus, the presence of *T. pseudospiralis* in this carnivore does not raise immediate public health concerns. As well, *T. pseudospiralis* is susceptible to freezing, lessening the likelihood of successful establishment in the Arctic, even if introduced with some regularity by migratory birds. Future regional surveillance efforts for *Trichinella* spp. could focus on wolverine (as a sentinel species), as well as omnivorous game animals (such as bear and wild boar), rodents, and carnivorous birds.

Conclusion

We report new host and geographic records for *T. pseudospiralis* in a wolverine, and the first report of this parasite on the mainland of Canada. This isolate was most closely related to those from Russia and Argentina, rather than populations of parasites at geographically proximate localities from North America. Distribution of *T. pseudospiralis* in subarctic Canada may

emphasize the potential role of migratory birds in long distance dispersal and potential introduction of non-native pathogens into remote regions. Additional field sampling is needed to elucidate whether *T. pseudospiralis* is established in the Canadian North or has been introduced transiently to the area via migratory birds. Our discovery, linked to explorations of parasite diversity emphasizes the importance of genetic identification of *Trichinella* using sequencing as well as the utility of monitoring for *Trichinella* spp. and other important food-borne parasites in wildlife, especially high trophic level carnivores and scavengers as an upstream measure of human risk.

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